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UNIVERSITY OF ALBERTA

Bacteriocin Production in *Carnobacterium piscicola* LV17 and Location of its Genetic Determinant

by

Cheol Ahn ©

A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF
Doctor of Philosophy
IN
Food Microbiology

Department of Food Science

EDMONTON, ALBERTA
Spring 1991

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submitted by **Cheol Ahn**

in partial fulfillment of the requirements for the

degree of **Doctor of Philosophy**

in **Food Microbiology**

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DEDICATION

To my wife, Young, and our parents

ABSTRACT

Strains of lactic acid bacteria isolated from vacuum-packaged meat, including *Carnobacterium piscicola* LV17, were screened for their bacteriocin-producing ability, and monitored for factors affecting bacteriocin production. Bacteriocins from *C. piscicola* LV17 were further studied in detail. They could be detected early in the logarithmic phase of growth in media with pH adjusted above 5.5. Partially purified bacteriocins from *C. piscicola* LV17 are active against closely related strains of lactic acid bacteria, some *Enterococcus* spp., and *Listeria monocytogenes*. The bacteriocins are proteinaceous, retained by dialysis tubing with molecular weight cut off of 6,000 to 8,000 dalton, and stable over a wide range of pH and during heat-treatment at 100°C for 30 min. The bacteriocins have a bactericidal mode of inhibition against *Carnobacterium divergens* LV13, act on the cytoplasmic membrane. Mutant studies revealed that bacteriocin production is mediated by two plasmids: 40 MDa (pCP40) and 49 MDa (pCP49) plasmids. Bacteriocin encoded by pCP49 is responsible for 80% of total bacteriocin activity, while bacteriocin encoded by pCP40 is responsible for 20% of activity. The two bacteriocins are different in their specificity.

The bacteriocin plasmids are non-conjugative, but can be co-mobilized by using the conjugative plasmid pAMβ1. Co-transfer of bacteriocin plasmids occurred in three ways: transfer of all three residential plasmids, transfer of pCP40 alone, and transfer of a co-integrate plasmid between pCP40 and pCP49. This was later resolved into two new plasmids with full bacteriocinogenicity. To develop a vector system, a chloramphenicol resistance plasmid (pCaT) from *Lactobacillus plantarum* caTC2R was separated from two other residential plasmids in this strain by using the pAMβ1-associated mobilization scheme. The plasmid pCaT was characterized by restriction enzyme mapping. Cloning experiments were done to determine the location of the chloramphenicol resistance gene on pCaT. A 2.6 Kb *Eco*RV-*Sal*I region of pCaT was demonstrated to contain the gene. Using pCaT as vector DNA for cloning of the bacteriocin gene from pCP49, a 9.6 Kb *Pst*I

fragment of pCP49 was demonstrated that encodes bacteriocin activity and immunity for the bacteriocin mediated by pCP49. Further subcloning of the 9.6 Kb fragment into another vector, pMG36e, showed that the 6.6 Kb *Pst*I-*Sph*I region is responsible for the regulatory elements and the structural gene for bacteriocin. Subsequently, the 3.0 Kb *Sph*I-*Pst*I region was shown to determine immunity to the bacteriocin.

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Table of Contents

Chapter Page

1. General Introduction ... 1

1.1. Lactic acid bacteria from meat ... 1

1.2. Antibiosis of lactic acid bacteria ... 3

1.3. Bacteriocins of lactic acid bacteria ... 4

1.4. Bacteriocins produced by *Carnobacterium piscicola* ... 6

1.5. References ... 9

2. Antibacterial Activity of Lactic Acid Bacteria Isolated from Vacuum-packaged Meats ... 15

2.1. Introduction ... 15

2.2. Materials and Methods ... 16

2.3. Results ... 19

2.4. Discussion ... 29

2.5. References ... 31

3. Plasmid-associated Bacteriocin Production by a Strain of *Carnobacterium piscicola* from Meat ... 34

3.1. Introduction ... 34

3.2. Materials and Methods ... 35

3.3. Results ... 39

3.4. Discussion ... 51

3.5. References ... 53

4. Mobilization of Plasmids Associated with Bacteriocin Production in *Carnobacterium piscicola* by pAMßl ... 56

4.1. Introduction ... 56

4.2. Materials and Methods ... 57

Chapter Page

4.3. Results 62

4.4. Discussion 73

4.5. References 76

5. **Mobilization and Location of the Genetic Determinant of Chloramphenicol Resistance from *Lactobacillus plantarum* caTC2R** ...80

5.1. Introduction 80

5.2. Materials and Methods 81

5.3. Results 85

5.4. Discussion 90

5.5. References 96

6. **Cloning of a 9.6 Kb *Pst*I Fragment Encoding Bacteriocin Production and Immunity from a Bacteriocin Plasmid of *Carnobacterium piscicola* LV17** 99

6.1. Introduction 99

6.2. Materials and Methods 100

6.3. Results 104

6.4. Discussion 118

6.5. References 121

7. **General Conclusions of the Study** 123

7.1. Physiological effects of bacteriocin production by *C. piscicola* LV17 123

7.2. Biochemical aspects of bacteriocins of *C. piscicola* LV17 124

7.3. Genetic aspects of bacteriocin production in *C. piscicola* LV17 125

7.4. Applicability of methods and products 126

7.5. Applicability of bacteriocins and bacteriocin producing strains to the food industry 127

7.6. References 128

List of Tables

Table Page

2.1 Growth characteristics of 10 selected lactic acid bacteria isolated from vacuum-packaged meats stored at 4°C 21

2.2 Fermentation patterns of selected lactic acid bacteria isolated from meats 22

2.3 Direct and deferred antagonism of ten bacteriocin 'producer' strains against nine lactic 'indicator' strains 23

2.4 Inhibitory activity of supernatant fluids from 'producer' strains of lactic acid bacteria isolated from meats grown in APT broth for up to 96 h 24

3.1 Indicator strains and their susceptibility to inhibitory substance produced by *Carnobacterium piscicola* LV17 determined by deferred inhibition technique on APT agar 40

3.2 Effect of heat and enzyme treatment on the activity of antagonistic substance produced by *C. piscicola* LV17 43

3.3 Effect of bacteriocin produced by *C. piscicola* LV17 on protoplasts of *C. divergens* LV13 45

4.1 Bacterial strains 58

4.2 Conjugation efficiency between *Streptococcus sanguis* Challis DL125 and *C. piscicola* LV17 64

4.3 Effect of added calcium on the stability of DNase I in the presence of selected proteases 68

4.4 Conjugation efficiencies between *C. piscicola* LV17.TC1001 and LV17.C2NZ 69

Table Page

5.1 Bacterial strains 82

5.2 Efficiency of conjugal transfer of pAMß1 from *S. sanguis* Challis DL125 to *L. plantarum* caTC2R and efficiency of co-transfer of pAMß1 and pCaT from *L. plantarum* caTC2R.pAM to *C. piscicola* LV17.C2 86

5.3 Efficiency of electrotransformation of pCaT into various strains of *Carnobacterium* and minimum inhibitory concentration (MIC) of chloramphenicol in each transformant 89

6.1 Bacterial strains and plasmids 101

6.2 Interactions between *C. piscicola* strains producing bacteriocin(s) encoded by pCP40 and(or) pCP49 106

6.3 Activity spectrum of cloned strain *C. piscicola* UAL26.CL97 containing a 25.5 Kb *Bgl*II fragment from a bacteriocin plasmid of *C. piscicola LV17* 110

6.4 Bacteriocin activity and immunity of subclones of a 25.5 Kb *Bgl*II fragment from pCP49 113

List of Figures

Figure Page

2.1 Growth, pH change and point at which antibacterial activity was first detected for lactic acid bacteria isolated from meats when grown in BM broth at 25°C 26

2.2 Mode of action of antibacterial substances produced by *Carnobacterium piscicola* strain UAL8 against indicator strain UAL9 27

2.3 Mode of action of antibacterial substances produced by an aciduric *Lactobacillus* strain UAL11 against indicator strain UAL9 28

3.1 Growth and production of bacteriocin by *C. piscicola* LV17 at 25°C in APT broth adjusted to initial pH 6.5 or 6.0 41

3.2 Mode of inhibition of bacteriocin produced by *C. piscicola* LV17 against *C. divergens* LV13 in phosphate buffer (50 mM, pH 7.0) 44

3.3 Plasmid profiles of *C. piscicola* LV17 and its mutants 47

3.4 Antagonistic activity of *C. piscicola* LV17 and its mutants 48

3.5 Digestion patterns of plasmid DNA isolated from *C. piscicola* LV17 and its mutants 50

4.1 Experimental scheme for mobilization of bacteriocin plasmids in *Carnobacterium piscicola* LV17 by pAMßl originated from *Streptococcus sanguis* Challis DL125 60

4.2 Plasmid profiles of the first and second stage transconjugants 65

4.3 Bacteriocin activity of transconjugants on the APT agar plates 66

4.4 *Bgl*II digestion pattern of plasmids from transconjugants, cointegrate, and destabilization product 71

5.1 Experimental scheme for mobilization of 8.5 Kb Cm^r plasmid pCAT by pAMß1 and separation of pCAT from pAMß1 by electrotransformation 83

5.2 Plasmid profiles of transconjugants and electrotransformants 87

Figure Page

5.3 Restriction map of 8.5 Kb Cm^r plasmid pCaT 91

5.4 Restriction analysis of cloned fragments from pCaT 92

5.5 Summary of cloning experiments and location of chloramphenicol acetyl transferase (CAT) gene in pCaT 93

6.1 Protoplast-induced curing of pCP9 from *C. piscicola* LV17.C33 containing pCP9 and pCP49 105

6.2 Restriction maps of a bacteriocin plasmid pCP49 from *C. piscicola* LV17, a religated plasmid pCP17 from a 25.5 Kb *Bgl*II fragment of pCP49, and a chloramphenicol resistance plasmid pCaT from *L. plantarum* caTC2R 107

6.3 Cloning of a 25.5 Kb *Bgl*II fragment of pCP49 into pCaT 109

6.4 *Bgl*II digestion pattern of plasmids originated from religated *Bgl* II fragments from pCP49 111

6.5 Cloning of a 16.7 Kb *Eco*RI fragment and a 9.6 Kb *Pst*I fragment of pCP17 using pCaT as vector DNA 112

6.6 Restriction map of pCaT300 containing a 9.6 Kb fragment from pCP17 115

6.7 Subcloning of a 9.6 Kb *Pst*I region of pCaT300 using pMG36e as vector DNA 116

6.8 Summary of cloning experiments 117

1. General Introduction

1.1. Lactic acid bacteria from meat

Lactic acid bacteria are a phylogenetically diverse group of microorganisms which have a common functional characteristic of converting carbohydrates into lactic acid as their major metabolic end product. They include the genera *Lactobacillus*, *Lactococcus*, *Pediococcus*, *Leuconostoc*, and *Streptococcus* in the *Clostridium* subdivision, and *Micrococcus*, *Propionibacterium*, and *Bifidobacterium* in the *Actinomycetes* subdivision of Gram-positive Eubacteria (44). Besides the nutritional benefit derived from the ability of lactic acid bacteria to ferment various carbohydrates, they have an inhibitory effect on other organisms as a result of their end product organic acids, with the associated drop in the pH of the fermentation medium. This has been exploited as the main principle in preservation of dairy, beverage, feed, and meat products. The high water activity of meat (a_w 0.96-0.97), and rich supply of nutrients, together with mildly acidic pH, provide excellent growth conditions not only for beneficial lactic acid bacteria, but also for spoilage microorganisms such as *Pseudomonas*, *Acinetobacter*, and *Alcaligenes*, or pathogenic microorganisms such as *Staphylococcus aureus*, *Salmonella* spp., *Clostridium botulinum*, and *Listeria monocytogenes* (20).

To preserve meat quality and safety, various methods have been applied, including cooling, drying, salting, fermentation and, more recently, vacuum or modified atmosphere packaging. As a result, proliferation and domination of lactic acid bacteria over the aerobic spoilage microflora occurs. Hammes *et al*. (12) reviewed the functional and ecological importance of the lactic acid bacteria in fermented sausage meat, including *Lactobacillus sake* and *Lactobacillus curvatus*. They showed the possible use of these lactic acid bacteria as meat starter cultures by specifying the desirable physiological properties of various strains.

In refrigerated, vacuum or modified atmosphere-packaged raw meat, evidence of domination of meat microflora by *Lactobacillus* and *Leuconostoc* species of lactic acid bacteria has been reported. Hitchner *et al.* (14) isolated 177 psychrotrophic lactic acid bacteria from vacuum-packaged beef. Of these, 159 strains were lactobacilli, and were classified as 'atypical' streptobacteria or betabacteria according to Orla-Jensen's criteria for classification (32). Holzapfel and Gerber (18) further classified one of the atypical betabacteria as *Lactobacillus divergens*, based upon genetic and biochemical data. Shaw and Harding (41) also isolated a variety of lactic acid bacteria from vacuum-packaged meats, and divided them into three clusters, including aciduric and nonaciduric lactobacilli and leuconostocs. One of the nonaciduric lactobacilli from cluster I was extensively compared with *Lactobacillus divergens* because of genetic and physiological similarities between them, and it was classified as *Lactobacillus carnis* based on differences in cellular fatty acid composition (42).

In the meantime, Hiu *et al.* (15) reported a new species of *Lactobacillus* isolated from salmonid fish, and named it *Lactobacillus piscicola*. However, the similar chemotaxonomic characteristics of these three new species were noticed by Collins *et al.* (5) and, based upon strong DNA-DNA homology data, they proposed a new genus *Carnobacterium*, combining *Lactobacillus carnis* and *Lactobacillus piscicola* into *Carnobacterium piscicola*. *C. piscicola*, therefore, is one of the traditional lactic acid bacteria, which is abundant in meat, and shares common properties with lactobacilli, including morphological and fermentation characteristics. However, the major distinctions from the genus *Lactobacillus* are its failure to grow on acetate agar (pH 5.6); predominance of L(+) lactate isomer; no detectable gas formation from glucose despite its heterofermentative mode of fermentation; and presence of oleic acid as its major $C_{18:1}$ fatty acid, instead of *cis*-vaccenic acid, which is common in *Lactobacillus* (42).

1.2. Antibiosis of lactic acid bacteria

Factors contributing to the antimicrobial effect or antibiosis of lactic acid bacteria against other microorganisms in fermentation media are (i) organic acids, (ii) CO_2, (iii) H_2O_2, (iv) diacetyl, and (v) bacteriocins. Lindgren and Dobrogosz (28) reviewed the effect of each of these factors, and assessed the importance of the concerted action of these factors for antibiosis *in vivo*. Of these, the antimicrobial effect of bacteriocins will be introduced in the following section (1.3).

The antimicrobial effect of organic acids, such as lactic and acetic acids, is due to the combined effect of lowered pH and undissociated forms of organic acids themselves (20). Low pH affects every aspect of cellular metabolism, retarding the growth of spoilage or pathogenic microorganisms in growth media. Undissociated lactic and acetic acids penetrate the cell membrane, and disturb the transmembrane potential, resulting in inhibition of substrate transport and membrane-bound F_0F_1 ATPase activity (30). The minimum inhibitory concentration of undissociated lactic acid (MIC undiss.) showed strain-specificity (28). *Lactococcus lactis* was shown not to be significantly affected by lowered pH in its membrane-bound ATPase activity (30).

CO_2 exerts its antimicrobial effect either by rendering the environment more anaerobic, or by inhibiting enzymatic decarboxylation, or by disrupting the cell membrane with the accumulation of the gaseous phase in the lipid bilayer (10, 20, 25).

The lethal effect of H_2O_2 may be due to the inactivation of essential biomolecules by the superoxide anion chain reaction (16). Production of H_2O_2 by lactic acid bacteria, and its bactericidal effect against food spoilage and pathogenic microorganisms has been widely reported (4, 23, 34, 50). The MIC for H_2O_2 against lactic acid bacteria was shown to be much higher than against *Staphylococcus aureus* (50). The mechanism whereby H_2O_2 activates the lactoperoxidase system (LPS) in dairy products is uncertain, but it seems to be linked to random oxidation of sulfhydryl groups of the biologically essential proteins by hypothiocyanate ion ($OSCN^-$). Increased lag phase by the action of LPS was observed in the case of *Listeria monocytogenes* Scott A (43).

The antibiotic effect of the aroma compound, diacetyl, is exerted mainly against Gram-negative bacteria because diacetyl is known to react specifically with arginine-binding proteins of Gram-negative origin (21).

1.3. Bacteriocins of lactic acid bacteria

In addition to the antimicrobial effect of the four different kinds of metabolic end products described above, many lactic acid bacteria produce antimicrobial substances known as bacteriocins or bacteriocin-like substances. In 1988, Klaenhammer (26) prepared an extensive review of the various groups of bacteriocins produced by lactic acid bacteria, and defined them as 'proteins or protein complexes with bactericidal activity directed against species that are usually closely related to the producer bacterium'. Among the bacteriocins reported so far, nisin is the best characterized. Extensive studies on its molecular structure, mechanism of action, and the genetic structure of the nisin gene have been published (2, 8, 9, 19, 24).

Production of most bacteriocins of lactic acid bacteria occurs in late logarithmic or stationary phases. However, lactostrepcin 2 from *Lactococcus lactis* 300 is produced in the early logarithmic phase (27), suggesting its association with primary functions of the cell. Inducibility of bacteriocins as in colicins of *Escherichia coli* (36) has not been reported in lactic acid bacteria.

The molecular structure of most bacteriocins of lactic acid bacteria is proteinaceous, in the form of polypeptides. Some bacteriocins are conjugated with lipids or carbohydrates (45). Molecular weights range from 3,500 dalton (Da), as in nisin, to 37,000 Da, as in helveticin J produced by *Lactobacillus helveticus* (22). Most of the bacteriocins are stable as multimers or aggregates. Amino acid sequence data for nisin showed the presence of three unusual amino acids: dehydroalanine, lanthionine, and β-methyl lanthionine, which are characteristic of antimicrobial compounds known as lantibiotics. Recently, two bacteriocins from *Leuconostoc* and *Lactobacillus* spp. were reported to have lantibiotic-like structure (13, 33). Most bacteriocins are stable to heating at 100°C for 10 min, and some bacteriocins, such as lactacin F from *Lactobacillus acidophilus* 88 (31), showed stability to autoclaving. Most bacteriocins are stable over a limited pH range.

The inhibitory spectrum of bacteriocins from lactic acid bacteria is narrow. They are generally active against closely related species. However, nisin and pediocin A from *Pediococcus pentosaceus* FBB61 and L-7230 (6) showed broader inhibitory spectra, including a wide range of Gram-positive foodborne pathogens such as *Clostridium botulinum* and *Listeria monocytogenes*.

The mode of action of most bacteriocins produced by lactic acid bacteria is bactericidal. However, lactocin 27 from *Lactobacillus helveticus* showed a bacteriostatic effect (45). The mechanism of action is either inhibition of macromolecular synthesis as in lactocin 27 (45) and diplococcin (7), or primary disruption of membrane function followed by inhibition of macromolecular synthesis as a secondary response, as in lactostrepcin 5 of a strain of *Lactococcus lactis* (51). Nisin inhibits function of the SH-containing membrane protein (19). Most bacteriocins are adsorbed nonspecifically to cell surfaces, but treatment of the cell surface with proteinase does not affect the binding of the bacteriocin.

The genetic determinant for bacteriocins produced by lactic acid bacteria is located on plasmids, as in the case of pediocin A with a 13.6 megadalton (MDa) plasmid (6), or on the chromosome as in the case of helveticin J (22) and lactacin B of *Lactobacillus acidophilus* N2 (1). However, most bacteriocins which showed plasmid association lacked further information on independent expression of the plasmid in heterospecies. Therefore, it is not certain whether a regulatory interaction exists between the plasmid and chromosome for bacteriocin production. The genetic determinant of some other bacteriocins, such as nisin, is located either on a plasmid or the chromosome, and can be transferred conjugally from the chromosome to recipient cells, indicating the presence of a transposon or an insertional sequence. Recently, the presence of an insertional sequence upstream of the nisin gene was reported (9), and the presence of a hot spot for integration of nisin into the chromosome was reported (35).

Genetic transfer of bacteriocin determinants has mainly been achieved by conjugation. Bacteriocin-producing ability for lactacin F, nisin, diplococcin, lactococcal bacteriocins, and pediocin A has been successfully transferred by conventional genetic methods, generating the possibility of *in vivo* combination of these bacteriocin plasmids in one host strain. For nonconjugative bacteriocin plasmids, co-mobilization with a conjugative plasmid such as pAMβ1 (3) has been suggested (11). Recently, electrotransformation has been successfully used in transforming homologous or heterologous genes or vectors into lactic acid bacteria (17, 29, 48).

Defining the genetic structure of bacteriocin genes by cloning is in the beginning stages. So far, the nisin gene is the first for which the genetic structure has been fully elucidated (9, 24). Van Belkum *et al.* (46, 47) cloned more than two bacteriocin genes from one bacteriocin plasmid of *Lactococcus lactis*, and Scherwitz-Harmon and McKay (38) cloned 3 *Bcl*I fragments from a bacteriocin plasmid of lactococcal origin. Recent developments in lactic vectors, including expression and integration vectors (37, 49), and availability of efficient transformation methods will facilitate genetic studies on bacteriocins of lactic acid bacteria.

1.4. Bacteriocins produced by *Carnobacterium piscicola*

Although *C. piscicola* is commonly isolated from vacuum- or modified atmosphere packaged meats, little is known about its ecological role in the microflora of anaerobically packaged meats. It is possible that bacteriocins help these organisms to dominate and become a major component of the meat microflora. In 1988, Schoebitz (40) inoculated *C. piscicola* onto the surface of meat, and found bacteriocin-like substances accumulated on the meat surface. In 1990, Schillinger and Holzapfel (39) reported initial screening results for bacteriocin production from the species within the genus *Carnobacterium*.

However, little is known about the mechanism of bacteriocin production by *C. piscicola*. If *C. piscicola* is a bacterium that has potential for use in meats as a 'starter' organism, i.e. an organism that may be introduced into meats as a competitive inhibitor of other spoilage and potentially pathogenic bacteria in meats, then more knowledge of the nature, function, and production of bacteriocins is required. In this study, the principal focus was the nature and genetic control of bacteriocin production by *C. piscicola* LV17. The work was divided into five main studies, which are outlined below.

The first part of the work (Chapter 2) describes results of screening experiments for bacteriocin-producing lactic acid bacteria from vacuum-packaged ground beef, including a comparison with *C. piscicola* LV17.

The second part of the work (Chapter 3) is devoted to the study of bacteriocin production by *C. piscicola* LV17, its mode of action, and biochemical characteristics of the partially-purified bacteriocin. Evidence of association of bacteriocinogenicity to two residential plasmids is presented.

In the third part of the work (Chapter 4), conjugal mobilization of the two non-conjugative plasmids associated with bacteriocin production in *C. piscicola* LV17 is achieved by introduction of the conjugative plasmid, pAMβ1. Electrotransformation of another marker plasmid into *C. piscicola* LV17, destabilization of the marker plasmids, and co-integrate formation between the two bacteriocin plasmids are described.

The fourth part of the work (Chapter 5) investigates the possible use of a chloramphenicol resistance plasmid from a strain of *Lactobacillus plantarum* as vector DNA for *Carnobacterium*. Isolation of the plasmid from other residential plasmids by another mobilization scheme, characterization by extensive restriction analysis, and cloning of the chloramphenicol resistance gene are shown.

The fifth part of the work (Chapter 6) gives the results of cloning of a 9.6 Kb fragment which encodes bacteriocin production and immunity from one of the two bacteriocin plasmids in *C. piscicola* LV17 using the chloramphenicol resistance plasmid as a vector. Expression of the plasmid-mediated bacteriocinogenicity of *C. piscicola* LV17 in other chromosomally-determined bacteriocin producer strains is described.

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2. Antibacterial Activity of Lactic Acid Bacteria Isolated from Vacuum-packaged Meats[1]

2.1. Introduction

Lactic acid bacteria have an ecological niche in refrigerated, vacuum-packaged fresh and processed meats. The increased use of modified atmosphere or vacuum packaging of meats has meant that there has been an increase in interest in these lactic acid bacteria, as evidenced by publications by Hitchener *et al.* (8), Holzapfel and Gerber (9), Shaw and Harding (20) and Schillinger and Lücke (15, 16). The lactic acid bacteria of meat are not as well defined as those used in dairy products or even those associated with vegetable fermentations, except for some specific starter organisms that are used in fermented meats.

Lactobacillus-type organisms from meats were generally described as 'not identifiable' or, based on Orla-Jensen's classification of the lactobacilli, as 'atypical streptobacteria' (19). Shaw and Harding (20) identified three principal groups of lactic acid bacteria among strains isolated from vacuum-packaged meats, including aciduric and non-aciduric *Lactobacillus*-type organisms and *Leuconostoc* species. New species of lactic acid bacteria from meats have been described, including *Lactobacillus divergens* (9) and *Lact. carnis* (21). These have subsequently been proposed as members of a new genus, as *Carnobacterium divergens* and *C. piscicola*, respectively (4). Schillinger and Lücke (16) developed a scheme for the identification of lactobacilli isolated from meats and described 10 species which commonly occur.

Although the predominance of lactic acid bacteria in vacuum-packaged meats markedly extends their refrigerated storage life, the meats ultimately spoil as a result of acidity or metabolic by-products produced by the lactic acid bacteria. The species or types of lactic acid bacteria which become predominant in vacuum-packaged meats can influence the storage life and acceptability of the product (16, 18). Factors that cause specific strains of lactic acid bacteria to dominate the microflora are not known, but bacteriocin production could play a role. These antibacterial substances are considered an important factor that influences the competitiveness of a bacterium in a fermenting ecosystem (11).

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[1] A version of this chapter has been published.
Ahn, C., and M.E. Stiles. 1990. Journal of Applied Bacteriology 69:302-310.

Bacteriocins are antibacterial substances which, based on knowledge of colicins and other bacteriocins, have been quite restrictively defined (23). In a review of the bacteriocins produced by lactic acid bacteria, Klaenhammer (11) defined them as 'proteins or protein complexes with bactericidal activity directed against species that are usually closely related to the producer bacterium'. Most knowledge of bacteriocins of lactic acid bacteria relates to lactococci and lactobacilli used as dairy starter bacteria. Bacteriocin production by some lactic acid bacteria associated with vegetable fermentations has also been reported (2, 5). Bacteriocin production may also be quite widespread among lactic acid bacteria from meats (10, 17, 24). In this study, lactic acid bacteria isolated from meats were screened for production of antibacterial compounds, and some characteristics of these substances were determined.

2.2. Materials and Methods

Sources and characteristics of lactic acid bacteria

Strains were obtained from studies of vacuum-packaged ground beef (3, 7), and from Dr. B.G. Shaw (AFRC, Institute of Food Research, Langford, Bristol, UK). Strains were screened for bacteriocin production by deferred inhibition (23). Selected strains were inoculated into cooked meat medium (Difco) and stored at 4°C for use as laboratory stock cultures; and into glycerol citrate medium and stored at -70°C. The strains were tested for growth in BM broth (MRS broth prepared without ammonium citrate and sodium acetate) at 1°, 4° and 15°C and the final pH in La broth (20) after incubation at 25°C for 7 d. Differentiation was based on criteria established by Shaw and Harding (20) and Schillinger and Lücke (16) including ability to produce gas from glucose; to grow on acetate agar adjusted to pH 5.6; to reduce 0.01% 2,3,5-triphenyltetrazolium chloride (TTC) in BM agar; and production of ammonia from arginine in a medium containing 0.05% (w/v) glucose.

All test samples were incubated anaerobically at 25°C and examined after 2 and 5 d. Carbohydrate fermentation tests were done by a microtechnique using Minitek well plates (BBL, Becton-Dickinson and Co.). A volume (0.5 ml) of the cells suspended in BM broth prepared without glucose and 0.5 ml of sterile 4% solutions of the sugars to be tested (see Table 2.2) were added to the wells and incubated anaerobically at 25°C for 18 h. Carbohydrate fermentation was tested with 0.04% bromocresol purple indicator.

Antibacterial activity

Strains were prepared for use by subculturing in BM or APT broth (Difco) at 25°C for 18-24 h not less than twice but not more than seven times. Potential producer strains were screened against a range of nine lactic 'indicator' strains: aciduric *Lactobacillus* meat strain (UAL3) (7); *C. divergens* LV13 (UAL9), *C. piscicola* LV17 (UAL8), *Lactobacillus* group II LV69 (UAL12), *Leuc. mesenteroides* ATCC (American Type Culture Collection) 23368 (UAL15), *Lact. plantarum* ATCC 4008 (UAL16), *Lact. viridescens* ATCC 12706 (UAL17), *Pediococcus acidilactici* ATCC 8042 (UAL18) and *Ped. parvulus* ATCC 1937 (UAL19). Producer strains were grown in BM broth at 25°C for 18 h and spot inoculated by replicator plating (Replicator Multiple Inoculator, KVL Laboratories, Cambridge, Ontario, Canada) on freshly prepared plates of APT, MRS and Tryptic Soy Agar (TSA; Difco). The inocula were allowed to dry and, based on methods reviewed by Tagg *et al.* (23), either [1] overlayered with 6.0 ml of the appropriate 'soft' agar (containing 0.75% agar) inoculated with an 'indicator' strain at 1 x 10^7 CFU/ml and incubated anaerobically at 25°C for 18 h (direct inhibition); or [2] incubated anaerobically for 18 h before being overlayered with the indicator strains (deferred inhibition). Inhibition was recorded as negative if no zone or a small indistinct zone of inhibition was observed around the 'producer' colony.

Ten 'producer' strains were selected for further study. Supernatant fluids from cultures grown in APT broth at 25°C for 12, 24, 40 and 96 h were obtained by centrifugation (5,000 g for 15 min). Sample supernatant fluids were either (a) unadjusted or (b) adjusted to pH 6.5 with NaOH (1, 5 and 10 N) and cells inactivated or removed by (c) heat treatment at 62°C for 30 min, (d) mixing with an equal volume of chloroform, or filtering through membrane filters, 0.45 µm pore size, either (e) Acrodisc (Gelman Sciences, Ann Arbor, Michigan) or (f) Millipore type HA. Supernatant fluids were stored at 1°C for subsequent testing by the spot on lawn or agar well techniques (22) against two selected indicator strains (UAL9, if appropriate, and one other strain that showed strong deferred antagonism). A volume (50 µl) of each supernatant fluid was placed on an APT plate that had been overlayered with 6 ml of APT soft agar (0.75% agar) containing 1 x 10^7 CFU of indicator cells/ml.

Subsequently, a sample that was active against an indicator strain was treated with protease from *Bacillus subtilis* (Sigma) and denatured proteinase (100°C for 3 min) at protease concentrations of 1 mg/ml of supernatant fluid; with 68 units of catalase (11,500 units/mg; Sigma) per ml; and heat-treated at 100°C for 30 min and 121°C for 15 min, and re-tested by the spot on lawn technique. Activity was also tested on inverted well plates (22) overlayered with the indicator strain.

Antibacterial substances were concentrated *ca.* 10 x in an Amicon ultrafiltration cell (Amicon Corp., Danvers, Massachusetts) with a 10,000 MW exclusion membrane under 40 psi nitrogen pressure. Activity of the retentate fluids was tested by the spot on lawn technique with 20 µl volumes of 1:1 serial dilutions of the supernatant fluids. Arbitrary activity units (AU) were determined from the greatest dilution that caused inhibition of the indicator lawn. Activity was also tested by agar well diffusion and spot on lawn techniques against a broader spectrum of bacteria including: *Acinetobacter calcoaceticus* ATCC 9036, *Alcaligenes viscosus* NCIB 8596, *B. cereus* ATCC 572, *B. subtilis* ATCC 737, *Enterococcus faecalis* ATCC 19433, *Ent. faecium* ATCC 19434, *Ent. faecium* (*durans*) ATCC 11576, *Listeria innocua* ATCC 33090, *L. monocytogenes* ATCC 15313, *Pseudomonas fluorescens* ATCC 13525, *Staphylococcus aureus* ATCC 25923, and *Escherichia coli* ATCC 11775.

Antimicrobial Action

The mode of inhibition of the antibacterial substances from producer strains UAL8 and UAL11 was determined with 100 AU of antibacterial substance added to APT broth inoculated with 1 x 10^6 CFU of indicator strain UAL9 per ml. Control samples were prepared without antibacterial substance or with protease added to give 1 mg of protease per ml of reaction initially, or after 90 min and 6 h incubation. Samples were held at 25°C and tested for viable count on APT agar at selected time intervals: every 30 min for the first 90 min; at 3 h intervals up to 9 h; and after 24 and 48 h. Sample dilutions were plated directly on APT or, after treatment with protease, incubated anaerobically at 25°C and counted after 24, 48 and 72 h.

2.3. Results

Ten of the lactic acid bacteria strains isolated from the vacuum-packaged meats produced antibacterial substances; some of these were active against several of the lactic acid bacteria used as indicator strains. Most inhibition was demonstrated by the deferred method on APT agar at pH 6.5. A few strains produced zones of inhibition when tested by the direct antagonism technique on APT agar. Very few strains produced marked zones of inhibition on TSA. Zones of inhibition on MRS plates were generally diffuse, lacking a clear line of demarcation between growth and no growth of the indicator strain. The pH of the growth medium and incubation temperature had a dramatic effect on production of antibacterial substances by the producer strains. At 30°C growth occurred without bacteriocin production, except for strains UAL11 and UAL89. APT agar at pH 6.5 was selected as the medium for study of antibacterial activity.

Ten 'producer' strains, including one of eight strains received from Dr. B.G. Shaw, were selected for further study. These strains and some of their growth characteristics are listed in Table 2.1 and their fermentation patterns for a range of carbohydrates are shown in Table 2.2. The strains did not ferment a wide range of carbohydrates, except UAL59 which utilized almost all of the carbohydrates tested. Based on the identification criteria proposed by Schillinger and Lücke (16), the most probable identity of strain UAL26 is *C. piscicola* (4). Strain UAL89 has a similar biotype but it produces a final pH 4.0 in La broth. Strains UAL4, UAL11, UAL72 and UAL86 are aciduric *Lactobacillus* spp., similar to *Lact. sake*. Strain UAL59 is also aciduric, but it is characteristic of *Lact. plantarum*. Strains UAL14 and UAL104 were shown to be *Leuconostoc* spp. by their gas production from glucose, growth on acetate agar at pH 5.6, failure to reduce TTC or to produce ammonia from arginine.

Most of the inhibitory activity detected for the producer strains was against closely related lactic acid bacteria. However, UAL8, UAL14, and UAL59 were also inhibitory to *Ent. faecalis*, *Ent. faecium* and *L. monocytogenes*. Only one strain, *Lact. plantarum* UAL59 showed activity against a broader spectrum of bacteria. The results for direct and deferred antagonism of the 10 producer strains against the selected indicator strains are shown in Table 2.3. Based on these results, appropriate indicator strains were selected to test the antibacterial activity of the treated cell supernatant fluids. From the results summarized in Table 2.4, it can be seen that there are marked differences in the characteristics of the antibacterial substances. Furthermore, the method used to inactivate the producer strain affects the activity of the antibacterial substance, for example, antibacterial substance produced by strain UAL104 was inactivated at 62°C; some antibacterial substances were inactivated by chloroform. Differences in inactivation by the membrane filters were attributed to differences in the protein-binding characteristics of the membranes. However, this did not explain the differences observed between indicator strains, for example producer strain UAL8 against indicator strains UAL9 and UAL17. The possibility exists that two or more antibacterial substances are produced by the strain, or that the indicator strains differ markedly in their susceptibility to the inhibitory substances.

Table 2.1. Growth characteristics of 10 selected lactic acid bacteria isolated from vacuum-packaged meats stored at 4°C.

Strain		Time (d) for visible growth in BM			Final pH La broth (7 d)
		15°C	4°C	1°C	
Lactobacillus sp.	UAL4	1	5	10	4.0
Carnobacterium piscicola	UAL8	1	4	9	4.7
Lactobacillus sp.	UAL11	1	6	NG	4.2
Leuconostoc sp.	UAL14	1	5	10	4.4
Carnobacterium piscicola	UAL26	1	4	8	4.7
Lactobacillus plantarum	UAL59	1	±	NG	3.7
Lactobacillus sp.	UAL72	1	5	10	3.7
Lactobacillus sp.	UAL86	1	4	7	3.7
Carnobacterium piscicola	UAL89	1	4	8	4.0
Leuconostoc sp.	UAL104	1	7	10	4.3

BM broth, Lactobacilli MRS broth prepared without ammonium citrate or sodium acetate; La broth, Lactobacilli MRS broth without phosphate buffer with 0.3% (w/v) sodium citrate replacing ammonium citrate and adjusted to pH 6.8; NG, no growth.

Table 2.2. Fermentation patterns of selected lactic acid bacteria isolated from meats.

	Strains of lactic acid bacteria[a]							
Carbohydrates	UAL4	UAL8	UAL11	UAL26	UAL59	UAL72	UAL86	UAL89
Amygdalin	-	+	-	+	+	-	-	-
Arabinose	+	-	+	-	+	+	+	-
Cellobiose	-	+	-	+	+	-	-	+
Galactose	+	-	+	-	+	+	+	-
Glucose	+	+	+	+	+	+	+	+
Glycerol	-	+	-	+	+	-	-	+
Lactose	-	-	-	-	+	-	-	-
Maltose	-	+	-	+	+	-	-	+
Mannitol	-	+	-	+	+	-	-	-
Melezitose	-	-	-	-	+	-	-	+
Raffinose	-	-	-	-	+	-	-	-
Rhamnose	-	-	-	-	+	-	-	-
Ribose	+	+	+	+	+	+	+	±
Salicin	-	+	+	+	+	-	-	+
Sorbitol	-	-	-	-	+	-	-	-
Sucrose	+	+	+	+	+	-	-	+
Trehalose	+	+	-	+	+	+	+	+
Xylose	-	-	-	-	±	-	-	-
Other tests								
K gluconate	+	-	-	-	+	+	+	-
Aesculin	-	+	+	+	+	-	-	+

[a] Identity of strains shown in Table 2.1.

Table 2.3. Direct and deferred antagonism of ten bacteriocin 'producer' strains against nine lactic 'indicator' strains.

Producer strains (UAL)[a]	Indicator strains[a]								
	3	8	9	12	15	16	17	18	19
4 dir[b]	-	-	-	-	-	-	-	-	-
def[b]	-	+	+	-	-	+	-	-	-
8 dir	-	-	+	-	-	-	+	+	-
def	+	-	+	+	-	+	+	+	+
11 dir	-	+	+	-	+	-	-	-	+
def	+	+	+	-	+	+	-	-	+
14 dir	+	+	-	-	-	+	+	+	-
def	+	+	+	+	+	+	+	+	+
26 dir	-	-	-	-	+	-	+	+	+
def	+	-	+	+	+	+	+	+	+
59 dir	-	-	-	-	-	-	-	+	-
def	+	+	+	-	-	+	-	+	+
72 dir	-	-	-	-	-	-	-	-	-
def	-	+	+	-	-	-	-	-	+
86 dir	-	-	-	-	-	-	-	-	-
def	-	+	+	-	-	-	-	-	+
89 dir	-	-	-	-	-	-	-	-	-
def	-	-	+	-	-	-	-	-	-
104 dir	+	-	-	-	-	-	+	+	-
def	+	+	+	+	+	+	+	+	+

[a] For key to indicator strains see text (page 17), and producer strains see Table 2.1.

[b] dir, direct antagonism; def, deferred antagonism.

Table 2.4. Inhibitory activity of supernatant fluids from 'producer' strains of lactic acid bacteria isolated from meats grown in APT broth for up to 96 h.

Producer strain[a]	Indicator strain[a]	Time of incubation[b]	Degree of inhibition by prepared supernatant fluids[c]					
			A	B	C	D	E	F
UAL4	UAL9	40	+	+	+	+	+	-
	UAL16	40	+	+	+	+	+	-
UAL8	UAL9	12-96[d]	+	+	+	+	+	+
	UAL17	40	+	+	+	±	+	-
UAL11	UAL9	12-96[d]	+	+	+	+	+	+
	UAL16	24	-	+	+	±	+	-
	UAL16	40	+	+	+	+	+	-
UAL14	UAL17	24	-	-	-	+	+	-
	UAL17	40	+	+	-	+	+	-
	UAL17	96	±	±	-	+	+	-
UAL26	UAL17	12	+	+	-	+	+	-
	UAL17	24	+	+	-	-	+	-
	UAL17	40	+	+	+	-	+	-
UAL59	UAL19	12	+	+	-	±	+	-
	UAL19	24	+	+	-	-	+	-
	UAL19	40	+	+	+	-	+	-
UAL72	UAL9	24	+	+	+	+	+	-
	UAL9	40	+	+	+	+	+	-
	UAL19	40	-	-	-	-	+	-
UAL86	UAL9	24	+	+	+	+	+	-
	UAL9	40	-	-	-	-	+	-
	UAL19	24	+	+	+	+	+	-
	UAL19	40	+	+	+	+	+	-
UAL89	UAL9	24	+	+	+	±	+	-
UAL104	UAL17	24	+	+	-	+	+	-
	UAL17	40	-	+	-	+	+	-

[a] For key to indicator strains see text, and producer strains see Table 2.1.

[b] Inhibition *not* observed at other test times.

[c] Treatment of supernatant fluids for activity testing: A, untreated; all other treatments adjusted to pH 6.5, and B, no additional treatment; C, heat-treated at 62°C for 30 min; D, mixed with an equal volume of chloroform; E, filtered through an Acrodisc filter, 0.45 μm; F, filtered through a Millipore filter, 0.45 μm.

[d] All supernatant fluids with all treatments tested positive at all testing times.

An active supernatant fluid from each producer strain was treated with catalase, 1 mg protease/ml of reaction mixture and heat-inactivated protease. In all cases, antagonism was eliminated by protease treatment, but not by catalase or heat-inactivated protease. The antibacterial activity of the producer strains was retained when plates prepared for the deferred inhibition test were inverted and overlayered with the indicator strain.

Production of antibacterial substances relative to growth of the cells is illustrated in Fig. 2.1. Activity for strains UAL8 and UAL11 was detected after incubation for 3 h at 25°C. Activity for the other strains was detected only after incubation for 9 h, in the late log phase of growth. At initial pH 5.6, non-aciduric strains UAL8, UAL26 and UAL89 grew but failed to produce their antibacterial substances. In some cases the antibacterial activity was lost during extended incubation of the cultures. Aciduric strains grew in BM broth adjusted to pH 5.5 or 5.0, but non-aciduric strains did not grow at pH 5.0.

Concentrated supernatant fluids of producer strains UAL8 and UAL11 were used to determine the mode of action of the bacteriocin-like substances against strain UAL9. Cells in APT or phosphate buffer, with inhibitory substance added to 100 AU/ml and protease (1 mg/ml) added at time 0, grew or survived in a manner identical to the control samples. The survival curves of strain UAL9 exposed to 100 AU inhibitory substance from UAL8 per ml of suspending medium are shown in Fig. 2.2. A bactericidal mode of action was interpreted from these results. Treatment of the sample dilutions with protease at the time of plating did not influence the number of surviving cells. Similarly, addition of protease to the suspending media after incubation for 90 min with inhibitory substance did not reverse the antibacterial effect. In contrast, the mode of action of the antibacterial substance produced by strain UAL11 is bacteriostatic, as illustrated by the survival curves in Fig. 2.3. When sample dilutions were treated with protease at the time of plating, cell counts remained almost constant over the 24 h treatment period. When samples were not treated with protease and plates were counted after incubation for 24 h, an apparent bactericidal effect was noted. This effect diminished when incubation of the plates was extended

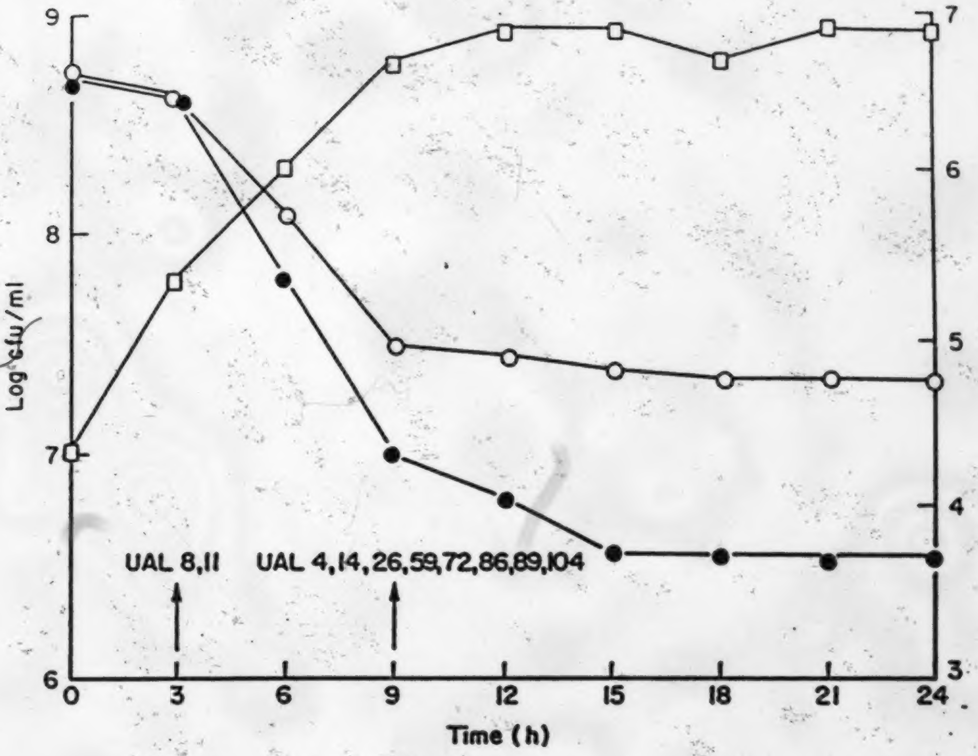

Figure 2.1. Growth, pH change and point at which antibacterial activity was first detected for lactic acid bacteria isolated from meats when grown in BM broth at 25°C.

□ Growth curve as represented by UAL8

● pH, aciduric strains as represented by UAL11

○ pH, non-aciduric strains as represented by UAL8

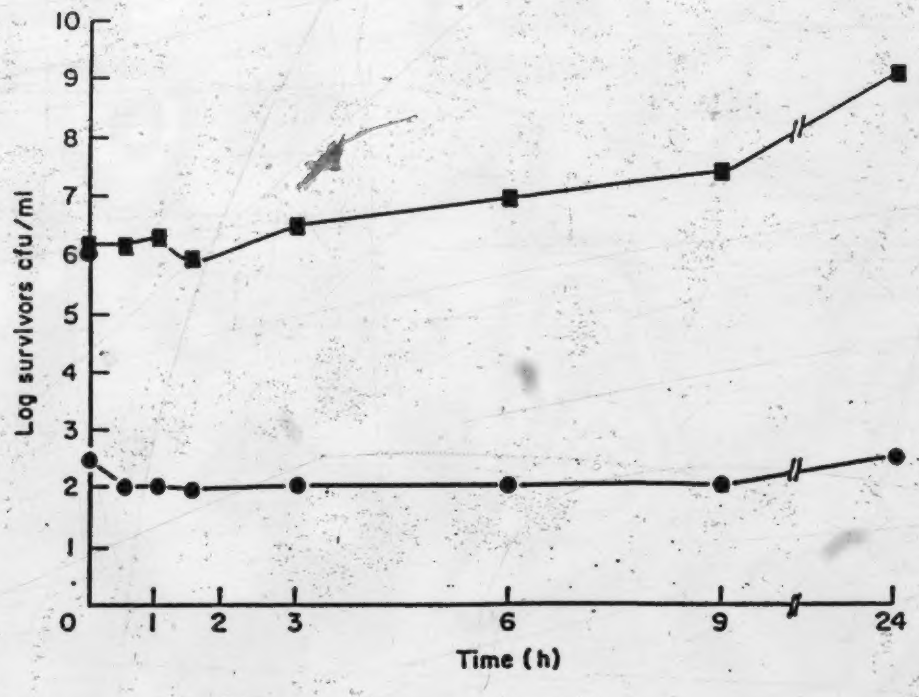

Figure 2.2. **Mode of action of antibacterial substances produced by *Carnobacterium piscicola* strain UAL8 against indicator strain UAL9.**

■ APT control ● APT + 100 AU bacteriocin

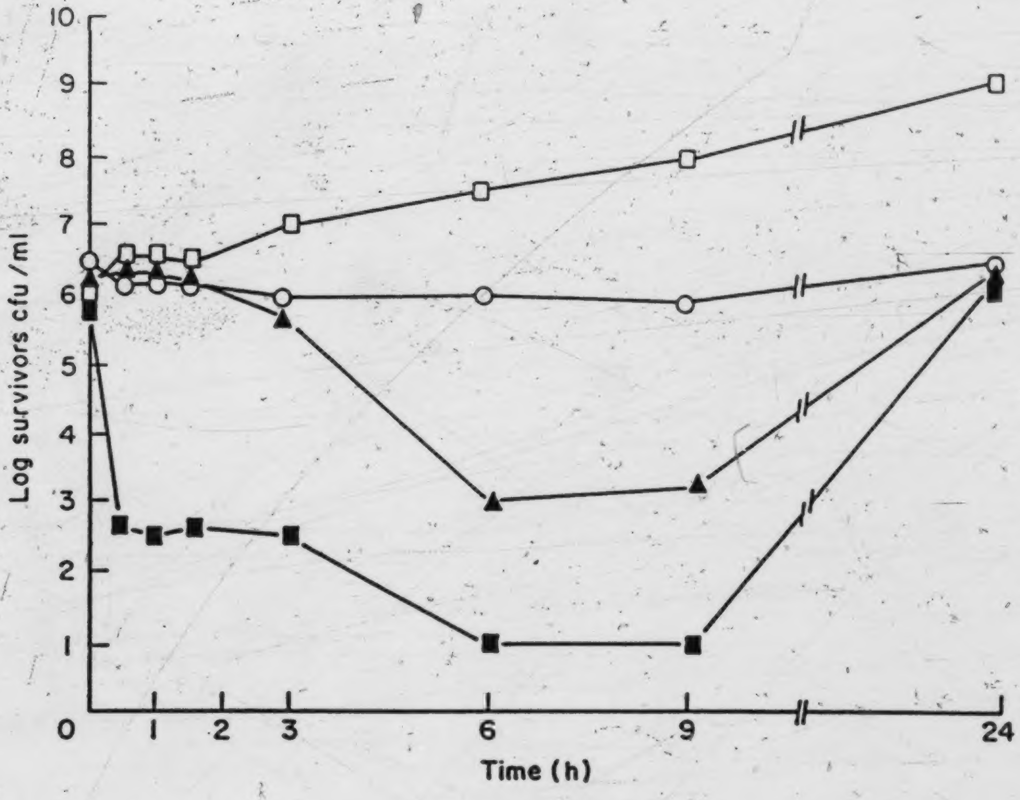

Figure 2.3. Mode of action of antibacterial substances produced by an aciduric *Lactobacillus* strain UAL11 against indicator strain UAL9.

☐	APT control
○	APT + 100 AU bacteriocin, inactivated with protease at time of plating; surviving colonies were counted after 24 h incubation on APT agar
■	APT + 100 AU bacteriocin, not inactivated with protease at time of plating; surviving colonies were counted after 24 h incubation on APT agar
▲	APT + 100 AU bacteriocin, not inactivated with protease at time of plating; surviving colonies were counted after 48 h incubation on APT agar

to 48 h, but remained constant after 48 h incubation. Treatment of the suspending media with protease after incubation for 90 min, 6 or 24 h with inhibitory substance reversed the antibacterial effect, and samples in APT broth were able to grow (data not shown).

2.4. Discussion

The predominance of lactic acid bacteria in the microflora of vacuum-packaged meats was originally documented by Pierson *et al.* (12). This has been confirmed by many studies, though the controlled growth of lactic acid bacteria in meats has only been exploited in fermented sausage meats. In laboratory studies that attempted to use lactic acid bacteria to extend the storage life of fresh, aerobically packaged meats (1, 6, 13, 14) mesophilic dairy starter cultures were generally used. Inoculation of 10^9 organisms/g was necessary to inhibit the development of the spoilage microflora. Schillinger and Lücke (15) studied the effect of an added lactic acid microflora that had been isolated from meats. They noted that little effect could be attributed to the inoculation of meats with 10^3-10^4 CFU/cm^2.

The lactic acid bacteria isolated from meats in our laboratory corresponded to the principal clusters reported by Shaw and Harding (20). The predominating strains of lactic acid bacteria isolated from vacuum-packaged ground beef varied between studies (3, 7). The strains isolated also differed in their ability to produce antibacterial substances. Growth medium influenced the production of antibacterial substances, and there was a notable pH effect on MRS medium adjusted to initial pH 5.6. Not all strains are likely to produce antibacterial substances under conditions that simulate the pH of normal meat (pH 5.5). Bacteriocin production has been cited as a factor promoting domination of a bacterial strain in a mixed fermentation (11).

Producer strains selected for this study had several attributes that should enable them to compete with the microflora of refrigerated meats. Many strains grow actively at 1°C and produce antibacterial substances active against a range of lactic acid bacteria.

Antibacterial activity of lactic acid bacteria can be caused by low pH, lactic acid, hydrogen peroxide, bacteriophage or bacteriocin-like compounds synthesized by the bacteria (23). The results of our studies eliminated the possibility that inhibition by the 'producer' strains was due to pH, hydrogen peroxide or bacteriophage. The antibacterial compounds are proteinaceous and bactericidal or bacteriostatic, as demonstrated in UAL8 or UAL11, respectively, as a result they are considered to be bacteriocin-like compounds (11). The time of bacteriocin production relative to growth of the cells varied between strains. Among the strains used in our studies, there are two organisms (UAL8 and UAL11) that produce antibacterial substances early in the growth cycle. This could be an important attribute facilitating the domination of these strains in a mixed fermentation, for example in a modified atmosphere-packaged meat.

It is not surprising that the mesophilic lactic acid bacteria used as dairy cultures are unable to dominate the microflora of meats unless they are inoculated at extremely high concentrations. It would seem, however, that lactic acid bacteria isolated from meats should be able to grow and dominate the meat microflora. The importance of the ability to grow at low temperature and low carbohydrate concentration is obvious, but the role of bacteriocin production as a factor in the ability of the bacteria to dominate the microflora of meats requires study.

2.5. References

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3. Plasmid-associated Bacteriocin Production by a Strain of *Carnobacterium piscicola* from Meat[1]

3.1. Introduction

Lactic acid bacteria in meats have acquired an importance far beyond their use as starter cultures for fermented sausages. This is the result of increased use of vacuum (modified atmosphere) packaged, chilled storage of meat and meat products in which lactic acid bacteria predominate and cause a natural fermentation. The formation of lactic and acetic acids and the associated drop in pH cause an antagonistic effect against other bacteria. This is supplemented in some lactic acid bacteria by production of antimicrobial compounds, such as bacteriocins and bacteriocin-like substances, which aid the domination of producer strains in mixed fermentations (12). The meat lactics have not been extensively studied, however, they are comprised of aciduric and nonaciduric *Lactobacillus*-type bacteria and *Leuconostoc* spp. (20, 22). The nonaciduric group of atypical lactobacilli are unable to grow on acetate agar adjusted to pH 5.6 (22).

Some heterofermentative strains of nonaciduric lactobacilli have been classified in a new genus *Carnobacterium*, based on their phenotypic characteristics (3). The new genus includes *C. divergens* described by Holzapfel and Gerber (8) and *C. piscicola* (3) described by Shaw and Harding (23) as *Lactobacillus carnis*. Predomination of these bacteria on vacuum packaged meats is not predicted, because the pH of normal meat drops rapidly to 5.6 during refrigerated storage. Their large numbers in meats, therefore, suggests an early competitive advantage as a result of rapid growth or production of inhibitory substance(s) early in the growth cycle. Production of a bacteriocin, sakacin A, by *Lactobacillus* sake isolated from meat has been reported (21). Production of bacteriocins or bacteriocin-like substances by a range of other meat isolates has also been reported by Ahn and Stiles (see

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[1] A version of this chapter has been published.
Ahn, C., and M.E. Stiles. 1990. Applied and Environmental Microbiology 56:2503-2510.

Chapter 2), notably with production of bacteriocin-like substances by strains of *C. piscicola* early in the growth phase.

This paper reports the production of bacteriocin by *C. piscicola* LV17 isolated from meat and the probable association of bacteriocin production with two plasmids contained in the cells.

3.2. Materials and Methods

Bacterial strains and growth media

C. piscicola LV17 and *C. divergens* LV13 isolated from meat by Dr. B.G. Shaw (Institute of Food Research, Langford, Bristol, U.K.) were used as bacteriocin producer and indicator strains, respectively. Other indicator strains are listed in Table 3.1 (page 40). Stock cultures were stored in Cooked Meat medium (Difco Laboratories Inc., Detroit, MI) at 4°C, and subcultured twice in BM broth (26) or APT broth (Difco) and incubated at 25°C for 24 h before use in experiments.

Bacteriocin detection and activity assay

Antagonistic substances produced by *C. piscicola* LV17 were detected by direct and deferred techniques (24) and confirmed by the spot-on-lawn method. Supernatant fluid from the producer strain was adjusted to pH 6.5 with 10 N NaOH and heated at 62°C for 30 min. A 50 µl aliquot was spotted onto the surface of a freshly prepared indicator lawn containing 10^7 CFU of indicator cells per ml in soft APT agar (0.75% agar); or placed in a 5 mm agar well, allowed to diffuse for 3 h and overlaid with an indicator lawn. Activity of bacteriocin was assayed by spotting serial dilutions of treated supernatant fluid onto an indicator lawn. The reciprocal of the greatest inhibitory dilution was used to calculate arbitrary activity units (AU) per ml.

Production of bacteriocin

C. piscicola LV17 was grown in APT broth adjusted to pH 6.5, 6.0, 5.5 and 5.0 with 5 N HCl. Inoculated broths were monitored for growth, pH change and bacteriocin production every 3 h for 24 h. Production of hydrogen peroxide by cultures grown on APT plates was checked by 2,2'-azino-di-(3-ethyl-benzthiazoline-6-sulfonic acid) (ABTS) test (15). Hydrogen peroxide action was differentiated from that of bacteriocin by inactivation of hydrogen peroxide with 68 units of catalase (Sigma Chem. Co., St. Louis, MO) per ml.

Partial purification of bacteriocin

The supernatant fluid from an 18 h culture of *C. piscicola* LV17 in APT broth was precipitated with ammonium sulfate (BDH Inc.), 65% saturation. The precipitate was collected by centrifugation, resuspended in phosphate buffer (50 mM, pH 7.0) and exhaustively dialyzed against the same phosphate buffer for 24 h at 4°C in a 6,000 to 8,000 MW cut-off dialysis bag (Spectra Por, Spectrum Medical Industries Inc., Los Angeles, CA). The retentate was filter sterilized (0.45µm, type HA filter, Millipore Corp., Bedford, MA) and stored at -70°C. Protein concentration was determined by microassay (Bio-Rad, Richmond, CA). Bacteriocin activity was determined against *C. divergens* LV13, as described above.

Sensitivity of bacteriocin to heat, enzyme activity and pH

Partially purified bacteriocin (6,400 AU/ml) was heated at 62 and 100°C for 30 min, and 121°C for 15 min and checked for residual activity. Inactivation of bacteriocin (6,400 AU/ml) by selected enzymes (1 mg/ml) was done in the buffers recommended by the supplier (Sigma). Reaction mixtures were incubated at 37 or 25°C for 60 min, heated at 95°C for 3 min and checked for bacteriocin activity. Enzymes included protease types I, IV, VIII (subtilisin Carlsberg), X (thermolysin), XIII, XIV (Pronase E), trypsin type IX, α- and β-chymotrypsin, pepsin, papain, protease-free α-amylase, lysozyme, lipase type VII, phospholipase C, DNase I and RNase A. All enzymes were purchased from Sigma.

Stability of partially purified bacteriocin at selected pH levels was determined by dialysis against buffers at pH 2.0 to 11.0, as described by Gonzalez and Kunka (7).

Adsorption studies

Adsorption of bacteriocin to sensitive and resistant cells was studied by adding 100 AU of partially purified bacteriocin to suspensions of 10^{10} cells per ml of phosphate buffer (50 mM, pH 7.0), using the procedures described by Andersson et al. (1). *Escherichia coli* ATCC 11225 and *C. piscicola* LV17 were used as resistant cells, and *C. divergens* LV13 and a nonbacteriocinogenic (Bac^-) strain of *C. piscicola* LV17 were used as sensitive cells.

Mode of inhibition

Partially purified bacteriocin was added to sterile phosphate buffer (50 mM, pH 7.0) containing 10^7 CFU of *C. divergens* LV13 per ml. Bacteriocin was added to give 12.5, 25 and 50 AU/ml and incubated at 25°C. Viable counts of *C. divergens* LV13 were determined on APT agar at selected time intervals. Cell lysis was checked by monitoring OD at 600 nm and confirmed microscopically. Indicator cells without bacteriocin and with Pronase E treated (1 mg/ml of reaction mixture; Sigma) bacteriocin were used as experimental controls. Pronase E (1 mg/ml of reaction mixture) was also added to cells treated with 25 AU bacteriocin/ml after 30 min reaction time.

Plasmid isolation

Miniscale extraction of plasmids from *C. piscicola* LV17 was done by modification of the Klaenhammer (11) method. A 1 ml aliquot of an overnight culture grown in APT broth at 25°C was used. The neutralizing salt solution proposed by Birnboim (2) was also used. Large scale extractions were done by 100x scaling up of the miniscale extraction process. The plasmids were further purified by CsCl density gradient ultracentrifugation (14). Agarose gel electrophoresis was done on 0.7% agarose gel (Pharmacia LKB Biotechnology 5-75182, Uppsala, Sweden) in TAE buffer at 60V for 3 h. Plasmid sizes were estimated using *Escherichia coli* strain V517 which contains plasmids of known size (13, 18).

Protoplast-induced curing

An overnight culture of *C. piscicola* LV17 was inoculated (1%) into 100 ml of M17 glucose broth (GM17;5). After incubation at 25°C for 3 h (OD 600nm 0.045), cells were harvested by centrifugation at 6000 x g, washed twice with distilled water, and resuspended in 10 ml of protoplasting buffer (PPB; 0.5M sucrose, 0.01 M Tris·Cl, pH 8.0). The cell suspension (5 ml) was treated with 5 ml of filter sterilized lysozyme solution (2 mg/ml in PPB; Sigma), following the procedure described by Gasson (6).

Another 5 ml of the cell suspension was diluted with 5 ml of PPB without lysozyme, and treated in the same manner as the lysozyme-treated cell suspension. Protoplast formation was confirmed by phase contrast microscopy and protoplasts (or cells) were harvested by centrifugation at 4000 x g, washed and resuspended in PPB (pH 7.0). The protoplast and cell suspensions were serially diluted for plating on GM17 agar and GM17 agar containing 0.5 M sucrose (SGM17). In some studies plates were overlaid with GM17 or SGM17 soft agar. Bacteriocin negative (Bac$^-$) or partial bacteriocin activity (Bac^p) colonies when screened against *C. divergens* LV13 were isolated and checked for their plasmid profiles. Bac$^-$ and Bac^p strains were screened for loss of other phenotypic attributes including: carbohydrate fermentation (22), antibiotic resistance, and resistance to a range of heavy metal ions (4) using procedures described by Novick and Roth (16). Digestion patterns of plasmids were determined with selected restriction enzymes, in particular *Bgl*II (Boehringer Mannheim Dorval, P.Q., Canada), using procedures recommended by the supplier(s). Estimation of plasmid DNA fragment sizes was done by multiple regression analysis (18) and used for calibration of molecular weight of plasmids, especially those plasmids larger than 40 MDa (megadaltons).

Effect of bacteriocin on protoplasts

Ten ml of protoplasted cell suspension of *C. divergens* LV13 was prepared as described above, and 50 AU/ml of partially purified bacteriocin was added. The protoplasts were incubated at 25°C for 90 min, centrifuged at 4000 x g, washed twice with PPB (pH 7.0) and appropriate dilutions were plated onto GM17 and SGM17 with soft agar overlays. Control cells without lysozyme treatment were also plated. The plates were incubated anaerobically at 25°C for 7 d for enumeration of colonies.

3.3. Results

The antimicrobial spectrum of the antagonistic substance produced by *C. piscicola* LV17 on APT agar plates against 15 Gram-positive bacteria is summarized in Table 3.1. This was confirmed using 100 AU of partially purified antagonistic substance by the spot-on-lawn technique. *C. piscicola* LV17 is active against other carnobacteria, lactobacilli including type strains and meat isolates, as well as some type strains of *Pediococcus*, *Enterococcus* spp. and *Listeria monocytogenes*. Antagonistic activity was not affected by catalase action and the ABTS test showed that hydrogen peroxide was not produced during aerobic or anaerobic growth of *C. piscicola* LV17 on APT agar plates. However, the antagonistic substance was not active against a range of Gram-negative bacteria including *Escherichia coli* ATCC 11775, *Klebsiella pneumoniae* ATCC 13883, *Salmonella typhimurium* ATCC 13311, *Serratia liquefaciens* ATCC 27592, *Acinetobacter calcoaceticus* ATCC 9036, and *Pseudomonas fluorescens* ATCC 13525.

Growth of *C. piscicola* LV17 in APT broth at pH 6.5 at 25°C resulted in a detectable amount of antagonistic substance in the supernatant fluid within 3 h, when starting with a population of approximately 5 x 10^7 CFU/ml. The amount of antagonistic substance in the supernatant fluid reached a maximum after 9 h, i.e. in the late logarithmic to early stationary phase of growth. The antagonistic substance remained active well into the stationary phase, but did not increase in concentration during the stationary phase, as

Table 3.1. Indicator strains and their susceptibility to inhibitory substance produced by *Carnobacterium piscicola* LV17 determined by deferred inhibition technique on APT agar[a].

Indicator strain	Wild Type Strain	Bac^p mutant[b] Strain C33	Bac^p mutant[b] Strain C6
Carnobacterium spp.			
C. piscicola LV17	-	-	-
C. piscicola UAL26	+	+	+
C. divergens LV13	+	+	+
Lactobacillus spp.			
L. plantarum ATCC 4008	-	-	-
L. sake ATCC 15521	+	+	-
L. viridens ATCC 12706	+	+	-
Aciduric *Lactobacillus*	+	+	-
Aciduric *Lactobacillus*	+	+	-
Pediococcus spp.			
P. acidilactici ATCC 8042	+	+	+
P. parvulus ATCC 1937	+	+	-
Enterococcus spp.			
E. faecalis ATCC 19433	+	+	-
E. faecium ATCC 19434	-	-	-
E. faecium (*durans*) ATCC 11576	+	+	-
Listeria spp.			
L. monocytogenes ATCC 15313	+	+	-
L. innocua ATCC 33090	-	-	-

[a] Plates incubated at 25°C overnight and clear zones with diameter >10 mm recorded as (+) sensitive, (-) resistant to bacteriocin.

[b] mutant with partial activity of wild type strain.

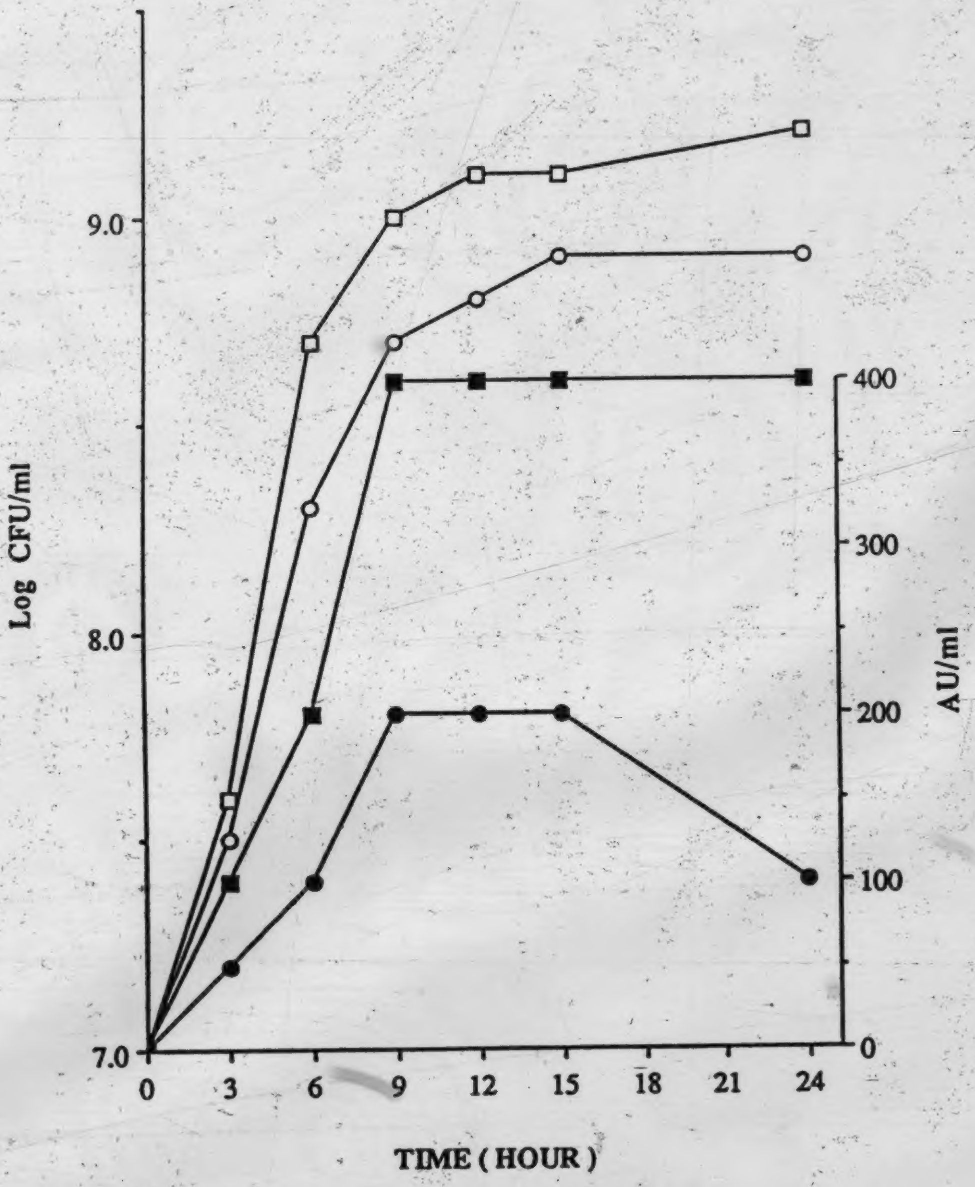


Fig. 3.1. **Growth and production of bacteriocin by *C. piscicola* LV17 at 25°C in APT broth adjusted to initial pH 6.5 or 6.0.**

Colony forming units per ml with initial pH 6.5 (□) and 6.0 (○); Arbitrary activity units (AU) of bacteriocin per ml produced by *C. piscicola* LV17 with initial pH 6.5 (■) and 6.0 (●).

indicated in Fig. 3.1. In broth adjusted to starting pH 6.0, production of antagonistic substance was reduced by 50%. At initial pH 5.5 and 5.0, growth of the organism occurred, but production of antagonistic substance was not detected at pH 5.0.

The antagonistic substance was stable to heat treatment at 62°C or boiling for 30 min, and 3% activity remained after autoclaving at 121°C for 15 min. Most proteolytic enzymes inactivated the antagonistic substance, except for pepsin and protease type XIII, as shown in Table 3.2. Some proteolytic enzymes caused partial inactivation, but nonproteolytic enzymes did not cause inactivation of the antagonistic substance. The antagonistic substance was stable over the pH range 2 to 11. Addition of bacteriocin (100 AU/ml) to suspensions of bacterial cells containing 10^{10} cells/ml resulted in a decrease in activity to 67 AU/ml within 30 min at 25°C, regardless of whether the cells are sensitive (*C. divergens* LV13 and Bac$^-$ mutant of *C. piscicola* LV17) or resistant (*C. piscicola* LV17 and *Escherichia coli*) or whether the cells were treated with protease or heated before addition of bacteriocin. The Bac$^-$ mutant and the wild type strain of *C. piscicola* LV17 caused the same amount of adsorption. The wild type strain did not produce a detectable amount of bacteriocin in 50 mM phosphate buffer when held at 25°C for 30 min.

Addition of antagonistic substance to a cell suspension of *C. divergens* LV13 at 2 x 10^7 CFU/ml in phosphate buffer (50 mM, pH 7.0) reduced the viable cell count >99.9% within 5 min in the presence of 50 AU of antagonistic substance per ml, and >99.9% within 30 min in the presence of 12.5 and 25 AU/ml, as illustrated in Fig. 3.2. Optical density did not change and cell lysis did not occur during the experiments. Addition of protease to the reaction mixture containing 25 AU of bacteriocin/ml immediately halted the lethal effect of the bacteriocin (see Fig. 3.2). After 24 h at 25°C, the number of viable cells in the 25 and 12.5 AU bacteriocin treatments had decreased to <1 x 10^0 and 8 x 10^0 CFU/ml, respectively. The protease-treated sample had a viable count of 8 x 10^3 CFU/ml. The bacteriocin from *C. piscicola* LV17 was also tested against protoplasts of *C. divergens* LV13. From the data in Table 3.3, it can be seen that protoplasts were killed by the

Table 3.2. Effect of heat and enzyme[a] treatment on the activity of antagonistic substance produced by *C. piscicola* LV17.

Treatment		Residual Activity (AU/ml)
Control		6400
62°C, 30 min		6400
100°C, 30 min		6400
121°C, 15 min		200
protease type	I	0
	IV	100
	VIII (subtilisin Calsberg)	0
	X (thermolysin)	0
	XIII	6400
	XIV (pronase E)	0
Trypsin (type IX)		0
α-chymotrypsin		0
β-chymotrypsin		50
pepsin		6400
papain (type IV)		0
α-amylase		6400
Lysozyme		6400
Lipase (type VII)		6400
Phospholipase C		6400
DNase I		6400
RNase A		6400

[a] Enzymes obtained from Sigma Chemical Co. and used at 1 mg/ml in the reaction mixture.

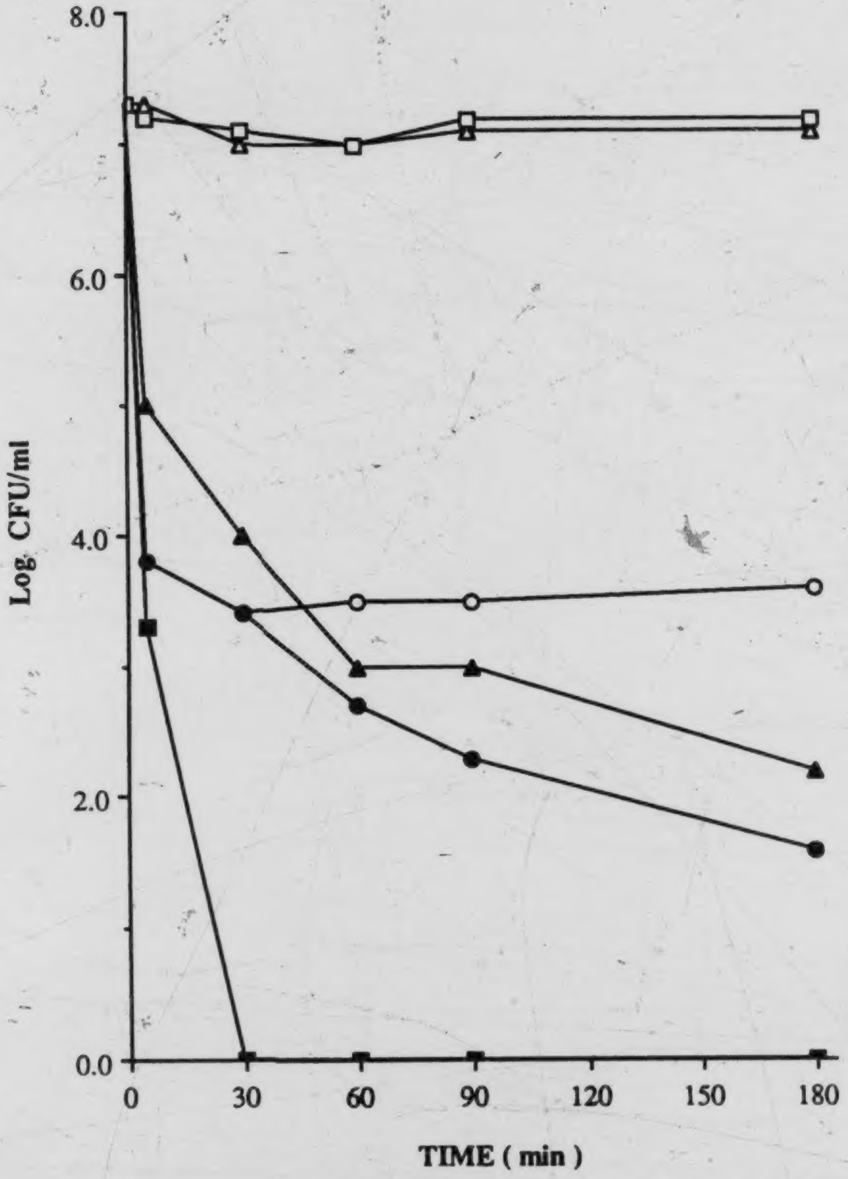


Fig. 3.2. Mode of inhibition of bacteriocin produced by *C. piscicola* LV17 against *C. divergens* LV13 in phosphate buffer (50 mM, pH 7.0).

Effects of partially purified bacteriocin concentrations of 50 AU/ml (■), 25 AU/ml (●), and 12.5 AU/ml (▲). Effect of addition of protease to reaction mixture containing 50 AU/ml at time 0 (□), and to 25 AU/ml after 30 min (○). Control cells without bacteriocin treatment (Δ).

Table 3.3. Effect of bacteriocin produced by *C. piscicola* LV17 on protoplasts of *C. divergens* LV13.

Bacteriocin Treatment	Protoplast formation[a]	Regeneration media[b]	CFU/ml[c]
Not treated	nonprotoplasted	GM17	4.8×10^9
		SGM17	4.7×10^9
	protoplasted	GM17	4.0×10^0
		SGM17	6.0×10^8
Treated	nonprotoplasted	GM17	1.0×10^4
		SGM17	2.0×10^4
	protoplasted	GM17	$< 1 \times 10^0$
		SGM17	$< 1 \times 10^0$

[a] Protoplasted cells treated with lysozyme (1 mg/ml) in protoplasting buffer (PPB; 0.5 M sucrose, 0.01 M Tris, pH 8.0); nonprotoplasted cells treated in PPB in the same manner as protoplasted cells, without lysozyme treatment.

[b] GM17, M17 glucose agar; SGM17, GM17 agar containing 0.5 M sucrose; both of GM17 and SGM17 agar plates were overlaid with GM17 and SGM17 soft agar (0.75% agar).

[c] Colony forming units per ml.

bacteriocin. The lethal effect of the bacteriocin against intact cells under high osmotic pressure was less than against the protoplasts. Without bacteriocin treatment, a total of 6 x 10^8 protoplasts was regenerated on the osmotically protected (SGM17) agar plates which were overlaid with SGM17 soft agar. This gave a regeneration rate of 12.8%. The bacteriocin treated (50 AU/ml) protoplasts were not regenerated when subjected to the same conditions.

The wild type strain of *C. piscicola* LV17 showed four plasmid bands (Fig. 3.3, lane 2) corresponding to 9.5, 23.5, 40 and 49 MDa based on multiple regression calibration (18) with plasmids from *E. coli* V517 (Fig. 3.3, lane 1). Protoplast-induced curing yielded 1.4% regenerated colonies on osmotically protected SGM17 agar. On the SGM17 agar plates overlaid with SGM17 soft agar, the regeneration rate reached 70%, but the curing effect was reduced. The regenerated colonies were screened for bacteriocin production against *C. divergens* LV13 and showed two phenotypes (Fig. 3.4, plate 4.1): (i) partial loss of bacteriocin activity (Bac^p, zones B and C) or (ii) complete loss of bacteriocin activity (Bac^-, zones D and E). The plasmid profiles of the Bac^p mutants C33 and C6 are shown in Fig. 3.3, lanes 3 and 4, respectively. Strain C33 had lost the 40 MDa plasmid which resulted in a 5% reduction in diameter of the activity zone against indicator strain *C. divergens* LV13 (Fig. 3.4.1, zone B). Strain C6 had lost the 49 MDa plasmid which resulted in a 50% reduction in diameter of the activity zone (Fig. 3.4.1, zone C). The Bac^- mutant strains C1 and C2 (Fig. 3.4.1, D and E) had lost both the 40 and 49 MDa plasmids or all of the plasmids, as shown in Fig. 3.3, lanes 5 and 6. During the screening of the plasmid profiles of Bac^+ colonies from protoplast induced curing, strain C5 was found which had bacteriocin activity (Fig. 3.4.1, zone F) equivalent to the wild type strain, but with different sizes of the larger plasmids, as shown in Fig. 3.3, lane 7. The molecular weights of the two large plasmids in strain C5 were 37 and 51 MDa, which suggested a possible rearrangement between the two large plasmids in the wild type strain.

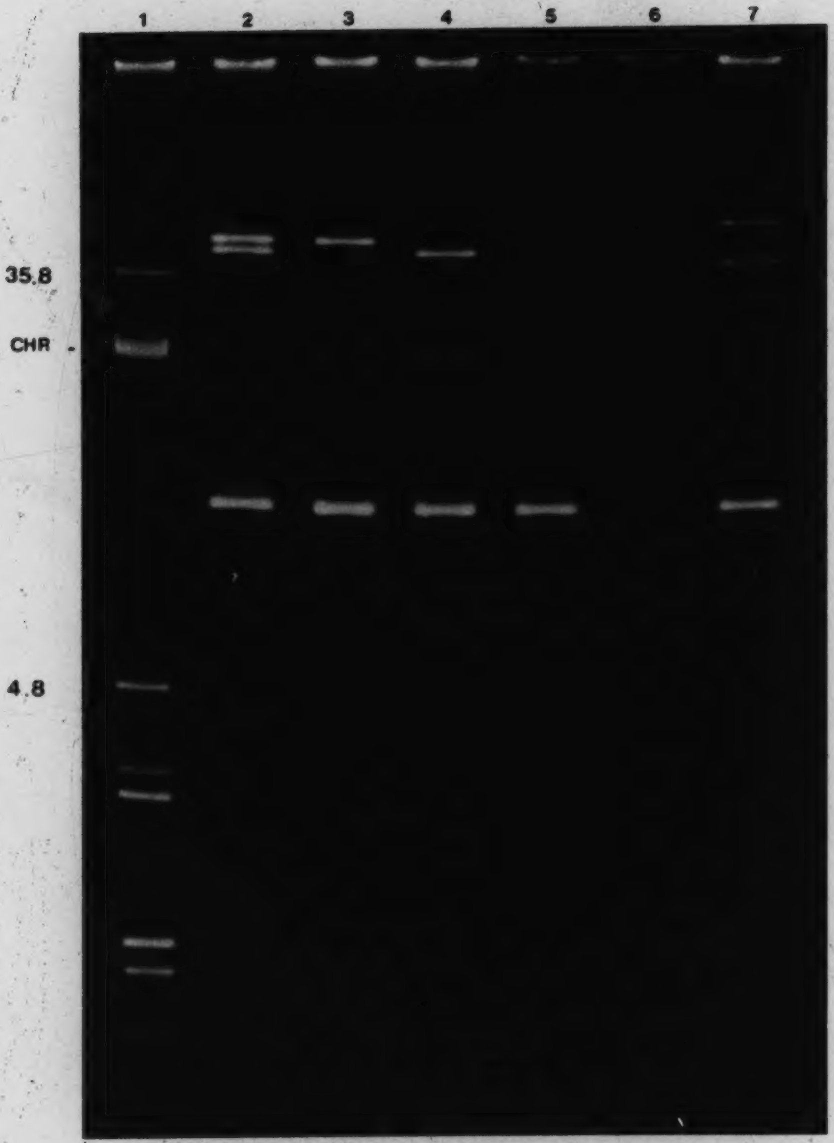
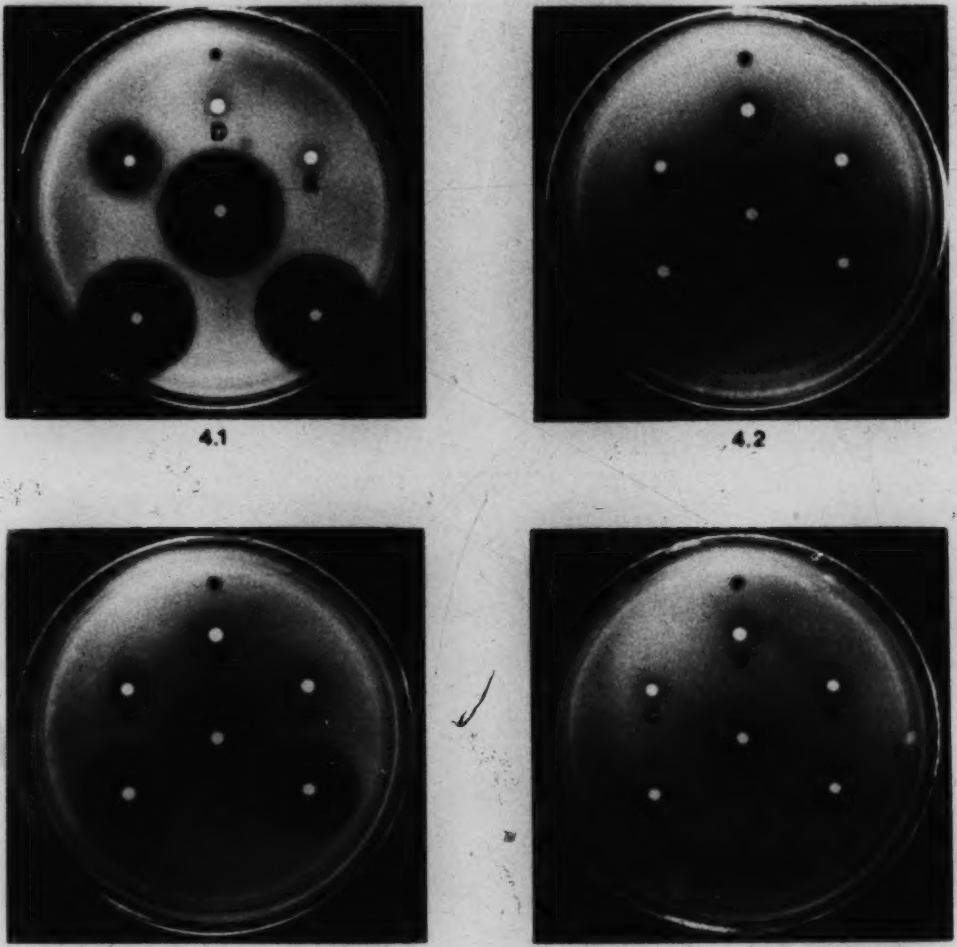


Fig. 3.3. Plasmid profiles of *C. piscicola* LV17 and its mutants.

Lane 1, *E. coli* V517 mobility standard with molecular sizes shown in MDa; lane 2, *C. piscicola* LV17 wild type; lane 3, Bac^p mutant strain C33 without 40 MDa plasmid; lane 4, Bac^p mutant strain C6 without 49 MDa plasmid; lane 5, Bac$^-$ mutant strain C1 without 40 and 49 MDa plasmids; lane 6, Bac$^-$ plasmidless mutant strain C2; lane 7, Bac$^+$ mutant strain C5 containing large plasmids with changed molecular weight; CHR, chromosome band.



4.1 4.2

4.3 4.4

Fig. 3.4. Antagonistic activity of *C. piscicola* LV17 and its mutants.

Overlaid with: 3.4.1. *C. piscicola* LV13; 3.4.2. Bac^p mutant strain C33 of *C. piscicola* LV17; 3.4.3. Bac^p mutant strain C6; and 3.4.4. wild type strain *C. piscicola* LV17 (A, wild type strain; B, Bac^p mutant strain C33; C, Bac^p mutant strain C6; D, Bac$^-$ mutant strain C1; E, Bac$^-$ plasmidless strain C2; F, Bac$^+$ strain C5).

Loss of the 40 MDa plasmid resulted in Bac^p strain C33 becoming partially sensitive to bacteriocin produced by the wild type strain, but insensitive to itself, as shown in Fig. 3.4.2, zones A and B. Loss of the 49 MDa plasmid resulted in Bac^p strain C6 becoming more sensitive to the wild type strain and the mutant strain C33, as shown by Fig. 3.4.3, zones A and B, while showing resistance to its own bacteriocin (zone C). The wild type strain was resistant to all of the mutant strains as well as to itself, as shown in Fig. 3.4.4.

*Bgl*II digestion patterns of the plasmid DNA from Bac^p strains C33 and C6, and Bac$^-$ strain C1 are shown in Fig. 3.5, lanes 3, 4 and 5, respectively. The 49 and 40 MDa plasmids resulted in five and four DNA fragments, respectively, and one 5.1 Kb fragment from each plasmid. However, the 9.5 and 23.5 MDa plasmids from the Bac$^-$ strain C1 resulted in four *Bgl*II fragments, and the sum of their molecular weights was only 9.5 MDa. The fact that the 23.5 MDa DNA band is most probably an open circular form of the 9.5 MDa plasmid was confirmed by two dimensional gel electrophoresis. This was shown by exposure of the gel to UV light for 5 min after the first separation. The *Bgl*II digestion pattern of Bac$^+$ strain C5 showed extinction of two fragments (15.4 and 25.5 Kb) compared with the wild type strain and appearance of two new fragments (11.2 and17.9 Kb), as illustrated in Fig. 3.5, lanes 2 and 6. The 6.8 Kb DNA band was judged to be two overlapping fragments, based on the intensity of the band.

All of the Bac^p and Bac$^-$ mutants, as well as the Bac$^+$ strain C5, showed the same physiological properties as those of the wild type strain, including fermentation pattern of 21 carbohydrates, and resistance patterns to 37 antibiotics and 20 heavy metal ions. No selection markers other than bacteriocin production and resistance to the bacteriocin were detected. The inhibitory spectrum of Bac^p strain C33 was the same as that of the wild type strain, as shown in Table 3.1. Bac^p strain C6 showed a very limited inhibitory spectrum.

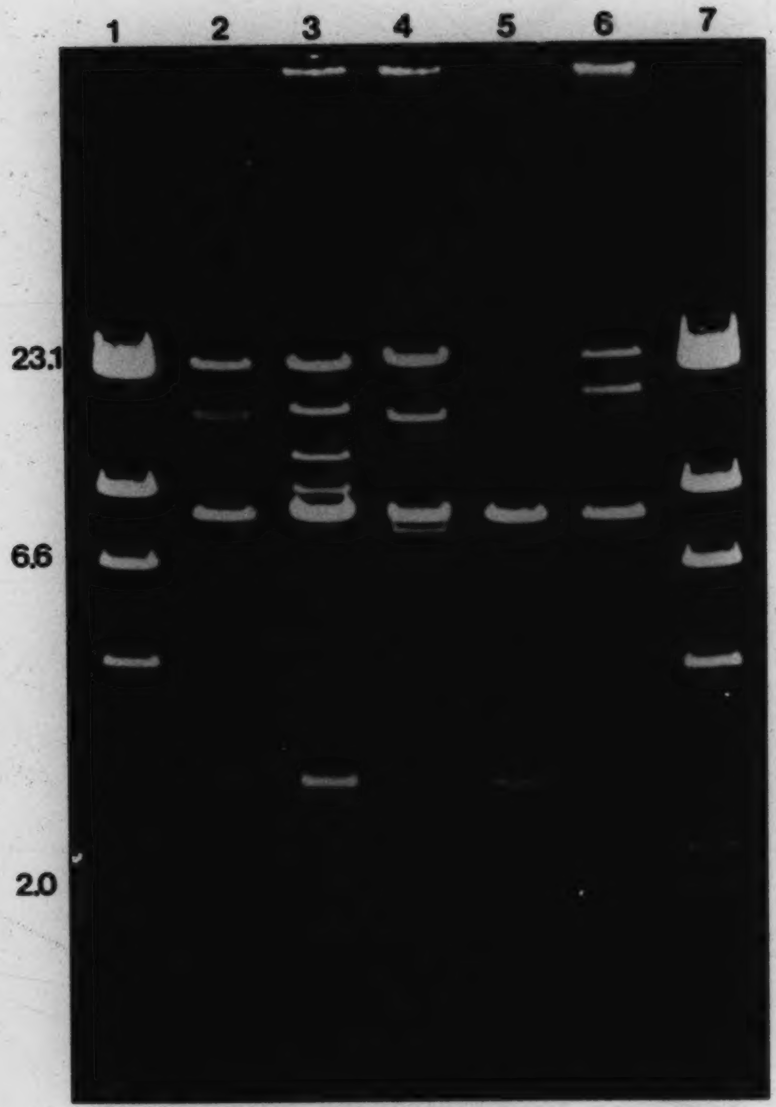


Fig. 3.5. Digestion patterns of plasmid DNA isolated from *C. piscicola* LV17 and its mutants.

Bacteriophage λ DNA digested with *Hin*dIII (lanes 1 and 7), *Bgl*II digests of plasmid DNA from wild type (lane 2), Bac^p mutant strain C33 (lane 3), Bac^p mutant strain C6 (lane 4), Bac$^-$ mutant strain C1 (lane 5), and Bac$^+$ mutant strain C5 (lane 6). Molecular sizes are shown in kilobases

3.4. Discussion

The antagonistic substance produced by *C. piscicola* LV17 was shown to be a bacteriocin, based on the criteria of various reviewers (12, 24). The substance is proteinaceous, with a bactericidal mode of action, and it is active against a relatively narrow range of Gram-positive bacteria. However, the antibacterial spectrum included some *Enterococcus* spp. and a strain of *Listeria monocytogenes*. Activity of bacteriocins produced by lactic acid bacteria against these genera was reported in Chapter 2. A large number of dairy cultures have been reported to produce bacteriocins and bacteriocin-like substances (12). *L. sake* isolated from meat produces sakacin A (21). Pediococci from fermented meats have also been reported to produce bacteriocins (9, 17) with activity against other Gram-positive bacteria (17). The possibility that antagonism was due to pH, organic acid ions, hydrogen peroxide or phage was carefully eliminated.

The bacteriocin produced by *C. piscicola* LV17 was detected early in the logarithmic phase of growth. This contrasts markedly with production of sakacin A by *L. sake* (21). Detection of early production could be a concentration effect in a highly sensitive indicator strain. Alternatively, it could represent genetic control that mediates bacteriocin production as a growth associated phenomenon. Early production of bacteriocin could be an important factor helping the producer strain to dominate a mixed population of closely related bacteria. Furthermore, absence of a homologous protease and heat stability of the bacteriocin could enable a starter culture to predominate in a mixed natural fermentation. Stability of the bacteriocin produced by *C. piscicola* over a wide range of pH was surprising, because the organism does not produce bacteriocin at pH below 5.5. The bacteriocin activity may be restored during assay on APT agar. This differs from nisin which is irreversibly denatured at high pH (10).

The bacteriocin of *C. piscicola* LV17 was nonspecifically adsorbed to cells, regardless of sensitivity, viability or protease treatment of the cells, a phenomenon also reported for pediocin PA-1 (7) and lactocin 27 (25). Therefore, the elimination of the inhibitory effect by addition of protease to bacteriocin-treated cells seems to be due to the proteolysis of free bacteriocin in the medium or bacteriocins bound to nonproteinaceous receptors on the cell surface. Activity of the bacteriocin against protoplasts suggests that the lethal effect is exerted directly on the cytoplasmic membrane (24). However, the reduced lethal effect against cells in hypertonic solution shows that contact between cell wall and cytoplasmic membrane might be necessary, probably for efficient transmission or translocation of the lethal factor.

Mutant studies revealed that both the 40 and 49 MDa plasmids are associated with bacteriocin production and resistance to the bacteriocin. The 49 MDa plasmid is responsible for approximately 80% of the bacteriocin activity, while the 40 MDa plasmid is responsible for only 20% of the activity. This is based on the size of activity zones and activity units of partially purified bacteriocin of each mutant grown under the same conditions. However, the nature of the association of these two plasmids with bacteriocin production and resistance is not clear. Further study is necessary to determine whether the two plasmids encode two different bacteriocins, or that one has a regulatory effect on the other, or that both of them regulate production of a chromosomally-determined bacteriocin. Initial indications are that two different bacteriocins are produced. The cause of the molecular weight change in the plasmids of the Bac$^+$ strain C5 is not certain. Scherwitz *et al.* (19) reported the appearance of a new 65 MDa plasmid from 33 and 88 MDa plasmids with concomitant loss of bacteriocin production resulting during conjugation of *Lactococcus lactis* subsp. *diacetylactis* WM$_4$. Further study is necessary to determine whether the change in plasmid size in *C. piscicola* LV17 is due to homology-dependent recombination occurring between 40 and 49 MDa plasmids or due to action of transposon-like unit on either of the plasmids.

3.5. References

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4. Mobilization of Plasmids Associated with Bacteriocin Production in *Carnobacterium piscicola* by pAMß1[1]

4.1. Introduction

Carnobacterium piscicola is a heterofermentative, nonaciduric bacterium that is frequently found in vacuum (modified atmosphere) packaged meat and poultry. It was differentiated from the *Lactobacillus* spp. found on these foods because of its failure to grow on acetate agar and other distinguishing phenotypic characteristics (6, 24). Its predominance on refrigerated, vacuum packaged meat suggests a competitive advantage over the initial microflora of meat. Previous reports by Ahn and Stiles (1, 2) showed that *C. piscicola* LV17 produces bacteriocin in the early stages of growth. Bacteriocin production is associated with 40 and 49 MDa plasmids contained in *C. piscicola* LV17. The 40 MDa plasmid (pCP40) is responsible for 20% of bacteriocin production, while the 49 MDa plasmid (pCP49) contributes 80% of the activity of the wild type strain. The bacteriocins are active against other lactic acid bacteria and strains of *Enterococcus faecalis* and *Listeria monocytogenes*, which suggests a potential use to improve meat starter cultures, as proposed for nisin (10).

Conjugal transfer of desired traits into other strains has been used as a starting point for improvement of starter cultures (8). Thus far, bacteriocinogenicity has been transferred to plasmidless mutant strains or nonbacteriocinogenic (Bac$^-$) heterospecies by conjugation (18, 21, 29). However, preliminary work showed that the two plasmids associated with bacteriocin production in *C. piscicola* LV17 are nonconjugative. To circumvent this, alternate mobilization systems to move nonconjugative plasmids into target cells have been surveyed. The conjugative plasmid pAMß1 (5) of 26.5 Kb size with macrolide-lincosamide-streptogramin B (MLS) resistance was selected as a mobilizing plasmid. pAMß1 has a wide host range in gram positive bacteria, including lactobacilli, lactococci, and streptococci (8). It has been used for mobilizing resident plasmids in a number of species (8) and for the mobilization of the nonconjugative proteinase plasmids from *Lactococcus lactis* subsp. *lactis* UC317 to *L. lactis* subsp. *cremoris* UC205 (9).

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[1] A version of this chapter has been submitted for publication.
Ahn, C., and M.E. Stiles. Applied and Environmental Microbiology.

This study reports the mobilization of two nonconjugative plasmids associated with bacteriocin production in *C. piscicola* LV17 by pAMß1 into a Bac$^-$ plasmidless mutant of the same organism.

4.2. Materials and Methods

Bacterial strains and media

Bacterial strains used in this study are listed in Table 4.1. *Carnobacterium piscicola* LV17 and *C. divergens* LV13 isolated from vacuum packaged meat by B.G. Shaw (Institute of Food Research, Langford, Bristol, U.K.) were used as bacteriocin producer (Bac$^+$) and indicator strains, respectively. *Streptococcus sanguis* Challis DL125 containing the 26.5 Kb conjugative plasmid pAMß1 was obtained from D.J. Leblanc (Laboratory of Molecular Biology, National Institute of Allergy and Infectious Diseases, Fort Detrik, Frederik, MD) and *Escherichia coli* MC1061 containing the plasmid pNZ12 (26) was obtained from W.M. de Vos (Netherlands Institute for Dairy Research, Ede, Holland). All of the strains of *Carnobacterium* and *Enterococcus faecalis* were subcultured in APT broth (Difco Laboratories Inc., Detroit, MI) at least three times before use, and *E. coli* MC1061 was grown in LB broth (23).

Bacteriocin detection and assay

Antagonistic activity of *C. piscicola* LV17 and its transconjugants against *C. divergens* LV13 was determined by deferred and spot-on-lawn methods (28). Activity was measured by serial dilution technique (2).

Table 4.1. Bacterial strains.

Strain	Phenotype[a]	Plasmids[b]	References
C. piscicola LV17			
Wild type	Cx^r, Suc$^+$, Gal$^-$ Bac$^+$, Em^s, Cm^s	pCP9, 40, 49	1,2,24
C2	Bac$^-$, Em^s, Cm^s	Plasmidless	2
C2 NZ	Bac$^-$, Em^s, Cm^r	pNZ12d	This study
TC1001	Bac$^+$, Em^r, Cm^s	pCP9, 40, 49, pAMβ1	This study
TC3001	Bac$^-$, Em^r, Cm^r	pNZ12d, pAMβ1	This study
TC3008	Bac^p, Em^r, Cm^r	pCP40, pNZ12d, pAMβ1	This study
TC3008M5	Bac^p, Em^s, Cm^s	pCP40	This study
TC3501	Bac$^+$, Em^r, Cm^r	pCP9, 40, 49, pNZ12d, pAMβ1	This study
TC3504	Bac$^+$, Em^r, Cm^r	pCP89, pNZ12d, pAMβ1	This study
TC3504M4	Bac$^+$, Em^r, Cm^s	pCP37, 51, pAMβ1	This study
C. divergens			
LV13	Bac$^-$	2.3 MDa plasmid	1,2,24
S. sanguis			
Challis DL125	Cx^s, Em^r, Suc$^-$, Gal$^+$	pAMβ1	13
E. coli			
MC1061	Cm^r	pNZ12	26

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[a] Bac, bacteriocin production; Cm, chloramphenicol; Cx, cloxacillin; Em, erythromycin; Suc, sucrose utilization; gal, galactose utilization; s, sensitive; r, resistant; +, positive; -, negative; p, partial production of bacteriocin; all strains of *C. piscicola* LV17 listed in Table 4.1 are Cx^r, Suc$^+$, Gal$^-$.

[b] pNZ12d, pNZ12 without *Bgl*II-sensitive site.

Plasmid isolation and digestion by restriction enzymes

Plasmid isolation was done by a modification of the Klaenhammer (11) and Birnboim (4) methods as described by Ahn and Stiles (2). Large scale extraction of plasmid DNA was done by 100x scale-up of the miniprep method and, if necessary, plasmids were further purified by CsCl density gradient ultracentrifugation (23). Digestion of plasmids by restriction enzymes (Boehringer Mannheim Canada, Laval, Québec) was done according to procedures recommended by the suppliers. Gel electrophoresis was done on 0.7% agarose gels (Pharmacia LKB Biotechnology 5-75182, Uppsala, Sweden) in TAE buffer at 5 V/cm for 3 h. In some cases, 0.9% agarose gels or a field strength of 3.3 V/cm were used for gel electrophoresis. Molecular weights of plasmids or fragments of plasmids were calculated by multiple regression analysis based on mobility standards from plasmids of *E. coli* V517 (15, 22) or *Hin*dIII digests of bacteriophage λ DNA (Boehringer Mannheim Canada).

Conjugation experiments

The experimental scheme for mobilizing the two plasmids associated with bacteriocin production is shown in Fig. 4.1. Conjugal transfer of pAMß1 to *C. piscicola* LV17 in the first stage conjugation was done by solid surface mating using *S. sanguis* Challis DL125 as donor and *C. piscicola* LV17 as recipient. A 0.2 ml volume of a 1:2 mixture of overnight cultures of donor and recipient cells in APT broth was placed on the surface of APT agar plates and incubated anaerobically at 25°C for 24 h. The cells were harvested by washing with 3 ml of APT broth and appropriate dilutions were plated onto APT screening agar plates containing 10 μg erythromycin (Em) and 10 μg cloxacillin (Cx, Sigma) per ml. Transconjugants were checked for bacteriocin (Bac) production, plasmid profiles, and fermentation of sucrose, trehalose, and galactose. Conjugation in a mating mixture treated with DNase I (Sigma; 1 mg/ml) and conjugation experiments between donor and filtrate of recipient cells and between recipient and filtrate of donor cells were done as experimental controls. The second stage conjugation was done between the

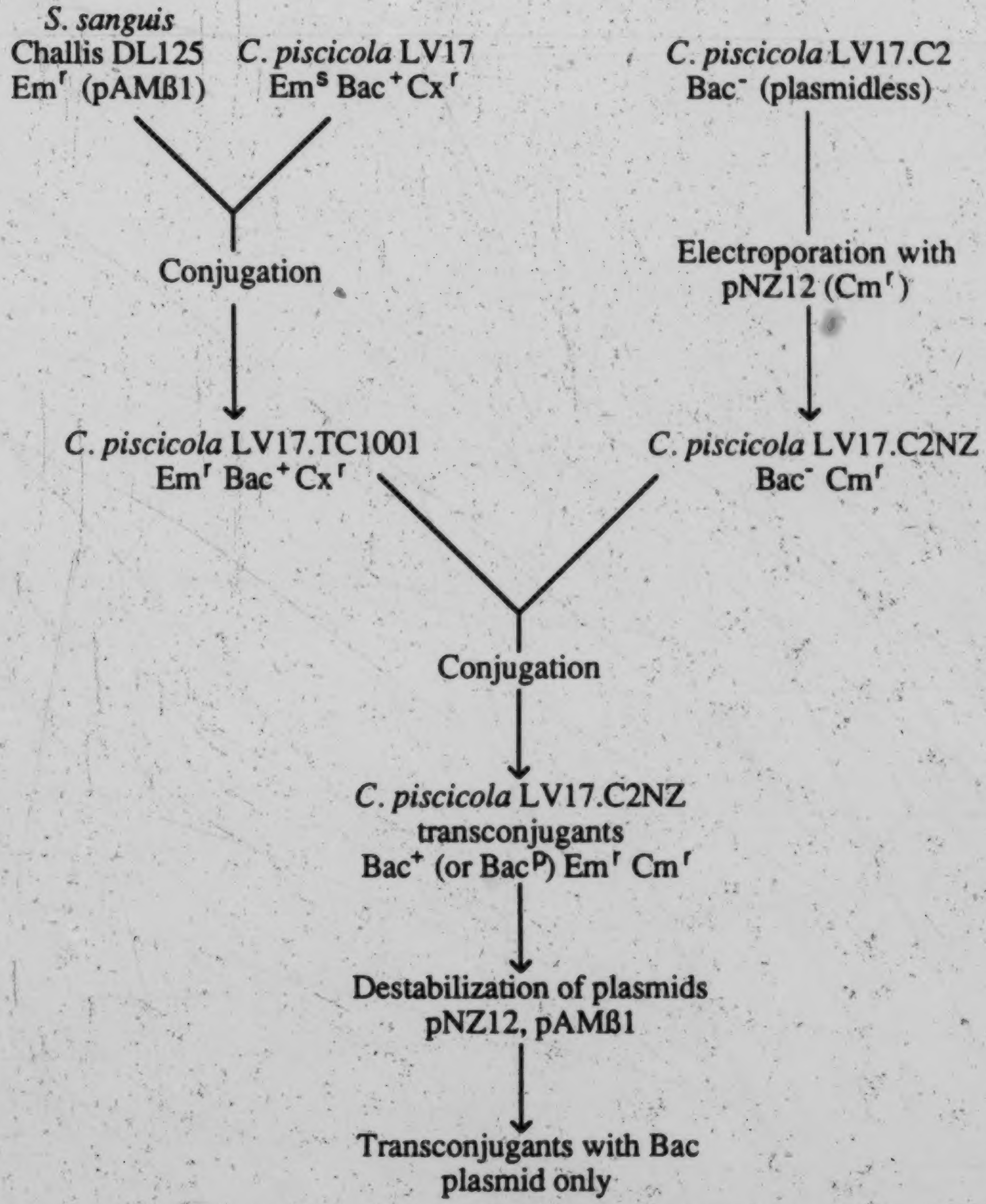
S. sanguis
Challis DL125
Em^r (pAMß1)
C. piscicola LV17
Em^s Bac^+ Cx^r
C. piscicola LV17.C2
Bac^- (plasmidless)
Conjugation
Electroporation with
pNZ12 (Cm^r)
C. piscicola LV17.TC1001
Em^r Bac^+ Cx^r
C. piscicola LV17.C2NZ
Bac^- Cm^r
Conjugation
C. piscicola LV17.C2NZ
transconjugants
Bac^+ (or Bac^p) Em^r Cm^r
Destabilization of plasmids
pNZ12, pAMß1
Transconjugants with Bac
plasmid only

Fig. 4.1. Experimental scheme for mobilization of bacteriocin plasmids in *Carnobacterium piscicola* LV17 by pAMßI originated from *Streptococcus sanguis* Challis DL125.

Bac, bacteriocin production; Cm, chloramphenicol; Em, erythromycin; +, bacteriocinogenic; p, partially bacteriocinogenic; -, non-bacteriocinogenic; r, resistant to antibiotic; s, sensitive to antibiotic.

pAMß1-containing (Em^r) *C. piscicola* LV17.TC1001 as donor and the chloramphenicol resistant (Cm^r), Bac^- mutant *C. piscicola* LV17.C2NZ as recipient, which was obtained by electrotransformation (see below). Conjugation between *C. piscicola* LV17.TC1001 and LV17.C2NZ was done by solid surface mating as described above, and filter mating. For filter mating, 0.2 ml of a 1:2 mixture of overnight cultures of donor and recipient cells was filtered through a sterile membrane (0.45 µm, type HA, Millipore Corp., Bedford, MA). The membrane was placed on APT agar, and incubated anaerobically at 25°C for 24 h. Cells were harvested by suspending the filter membrane in 3 ml of APT broth and appropriate dilutions were plated on APT screening plates containing Cm or Em (10 µg/ml), or both antibiotics and *C. piscicola* LV17 bacteriocin. These plates were prepared by overlayering an overnight lawn of *C. piscicola* LV17 on APT agar with agar containing double strength Cm and Em. Transconjugants were checked for Bac^+ and plasmid profiles. All mating mixtures were treated with Pronase E (Sigma, 1 mg/ml) and the DNase I-treated control contained Ca^{2+} (5mM) in the mating mixture to protect DNase I from digestion by Pronase E (see later).

Self-transmissibility of *C. piscicola* LV17 plasmids

Conjugation was done between *C. piscicola* LV17 as donor and a Cm^r, Bac^- mutant *C. piscicola* LV17.C2NZ, using solid surface and filter mating methods. Transconjugants were screened on APT agar plates containing 10 µg of Cm per ml and *C. piscicola* LV17 bacteriocin prepared by overlayering with APT agar containing double strength concentration of Cm, as described earlier.

Electrotransformation of pNZ12 into *C. piscicola* LV17.C2

The plasmidless mutant strain *C. piscicola* LV17C2 was identical in phenotype to the wild type strain, except for bacteriocin production (2). The plasmid pNZ12 encoding chloramphenicol resistance (26) was transformed into LV17.C2 by electrotransformation with a Gene Pulser ™ (Bio-Rad Laboratories, Richmond, CA) according to the protocol recommended by the manufacturer with HEPES-buffered sucrose electrotransformation buffer (HEB; 272 mM sucrose, 1 mM $MgCl_2$, 7 mM HEPES pH 7.4). A single pulse was delivered at 25 µFD and 200 ohms resistance at different voltages. Transformants were screened on APT agar plates containing 10 µg chloramphenicol per ml. Presence of pNZ12 in the transformants was confirmed by DNA extraction and digestion by restriction enzymes.

Stability of DNase I

Stability of DNase I in the presence of protease and Ca^{2+} (20) was checked by mixing equal amounts of DNase I (Sigma) with trypsin (Sigma), protease type I (Sigma), or Pronase E (Sigma), all at concentrations of 1 mg/ml, with or without 5 mM Ca^{2+} ($CaCl_2$), and placing the mixture on DNase Test Agar (Difco) plates. After washing the surface with 0.1 N HCl, appearance of clear zones was recorded as positive for DNase action.

Destabilization of marker plasmids

Transconjugant strains showing Bac^+ or Bac^p (partial production of bacteriocin) were cured of the antibiotic resistance plasmids by subculturing six consecutive times without Cm or Em (13) and screening for sensitivity to the antibiotics.

4.3. Results

Self-transmissibility of the bacteriocin plasmids pCP40 and pCP49 was checked by conjugation studies between *C. piscicola* LV17 and its plasmidless mutant with chloramphenicol resistance incorporated by electrotransformation of pNZ12. No transconjugants were obtained on the screening media containing chloramphenicol and bacteriocin produced by *C. piscicola* LV17. Therefore, under the conditions of this experiment it can be assumed that these plasmids are nonconjugative.

The first stage conjugation between erythromycin resistant (Em^r) *S. sanguis* Challis DL125 containing pAMß1 as donor and the cloxacillin resistant (Cx^r) bacteriocinogenic *C. piscicola* LV17 as recipient resulted in transfer of pAMß1 to *C. piscicola* with an efficiency of 10^{-6} transconjugants per donor, as illustrated in Table 4.2. Addition of DNase I (1 mg/ml) to the mating mixture did not affect the conjugation efficiency. Mating mixtures with donor cells and filtrate of the recipient cell culture or recipient cells and filtrate of donor cell culture did not give rise to transconjugants on the Cx-Em screening agar. The Cx^r and Em^r transconjugants were assumed to be *C. piscicola* LV17 because Cx^r is chromosomally determined in *C. piscicola* LV17 (2). This was confirmed by checking other chromosomal markers, including fermentation of sucrose and trehalose by *C. piscicola* LV17 and galactose by *S. sanguis*. All of the transconjugants fermented sucrose and trehalose on basal medium (see Chapter 2) containing either of the carbohydrates and both Cx and Em, but failed to ferment galactose. Plasmid profiles of the transconjugant LV17.TC1001 determined by agarose gel electrophoresis showed the presence of pAMß1 in addition to the original three plasmids: pCP9, pCP40, and pCP49 (Fig. 4.2-A, lanes 2 to 4). The bacteriocin activity and immunity of transconjugant TC1001 were not affected by the presence of pAMß1 (Fig. 4.3, zones a and c).

Electrotransformation of pNZ12 into the Bac$^-$ plasmidless mutant *C. piscicola* LV17.C2 to develop Cm^r as a selectable marker resulted in 74 transformants per µg of DNA at 6.25 kV/cm. The efficiency decreased to 4 transformants per µg of DNA when the field strength was reduced to 5 kV/cm, whereas no transformants were obtained at field strength of 3.75 kV/cm or less. Maximum efficiency of transformation occurred at 0.15 µg of DNA. Transformed pNZ12 showed the same restriction sites with *Sal*I, *Nco*I, and *Taq*I as observed with the original plasmid pNZ12, generating 4.3 Kb length. However, it was not digested by *Bgl*II and *Apa*I, which are located in the kanamycin resistance-encoding region of pNZ12, and designated as pNZ12d (Fig. 4.2-A, lane 5). Kanamycin resistance of transformants was the same as non-transformants on APT agar plate up to 256 µg per

Table 4.2. Conjugation efficiency between *S. sanguis* Challis DL125 and *C. piscicola* LV17.

DNase I Treatment	Donor CFU/ml	Transconjugants CFU/ml	Efficiency (transconjugants /donor)
Not treated	1.90×10^9	1.99×10^3	1.05×10^{-6}
Treated	1.18×10^9	2.32×10^3	1.97×10^{-6}

* All numerical values obtained are averages of two identical experiments

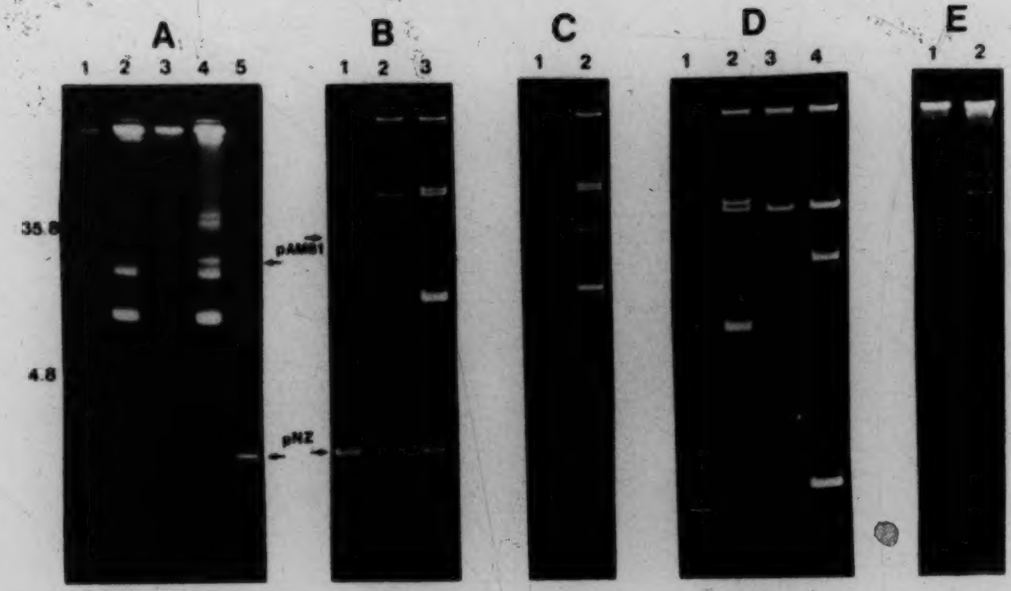

Fig. 4.2. Plasmid profiles of the first and second stage transconjugants.

A. Transfer of pAMßl to *C. piscicola* LV17 and electrotransformant of pNZ12. Lane 1, *E. coli* V517 as mobility standard; lane 2, *C. piscicola* LV17; lane 3, *S. sanguis* Challis DL125 containing pAMßl; lane 4, *C. piscicola* LV17.TC1001; lane 5, *C. piscicola* LV17.C2NZ, electrotransformant of pNZ12.

B. The second stage transconjugants. Lane 1, *C. piscicola* LV17.TC3001; lane 2, TC3008; lane 3, TC3501.

C. The second stage transconjugants. Lane 1, TC3504; lane 2, TC3501.

D. Destabilization of TC3008. Lane 1, *E. coli* V517 as mobility standard; lane 2, *C. piscicola* LV17 wild type; lane 3, TC3008M5; lane 4, TC3008.

E. Destabilization of TC3504. Lane 1, TC3504; lane 2, TC3504M4. A 0.7% agarose gel was used for gel electrophoresis at 5.0 V/cm. However, for gel electrophoresis shown in panel A, 0.9% agarose gel was used to differentiate pAMßl from the open circular form of pCP9 and chromosomal DNA band. pAMßl is marked by an arrow and molecular weight is expressed in megadaltons. See Table 4.1 for description of plasmid content of each strain.

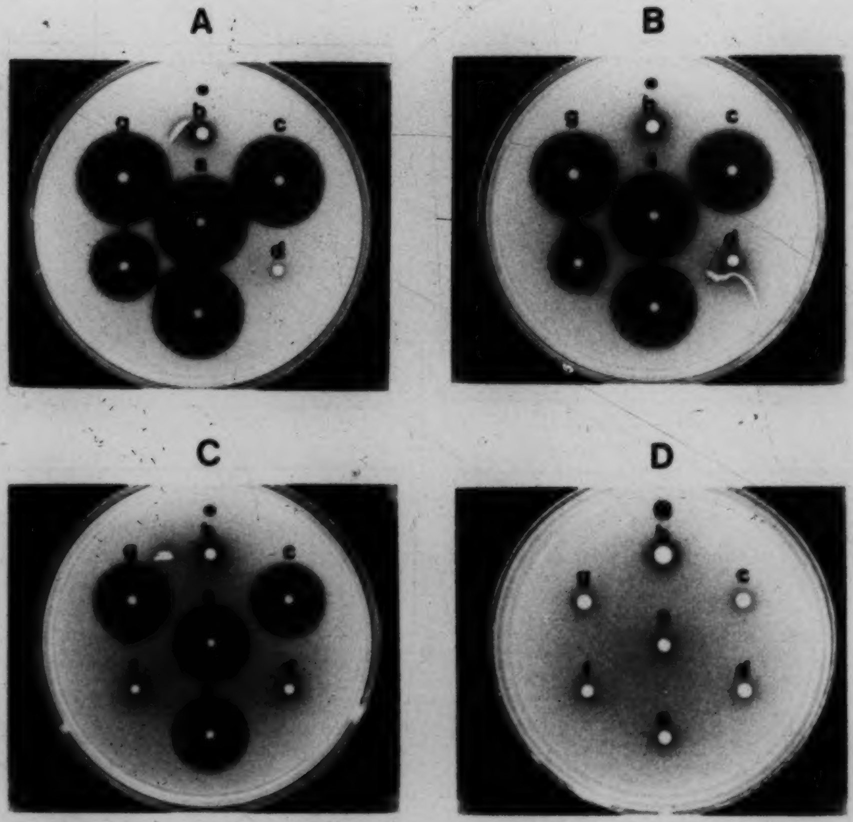

Fig. 4.3. Bacteriocin activity of transconjugants on the APT agar plates.

Overlayered with *C. divergens* LV13 (A); Bac$^-$ mutant *C. piscicola* LV17.C2 (B); Bac^p transconjugant TC3008 (C); Bac$^+$ wild type *C. piscicola* LV17 (D). Zone a, *C. piscicola* LV17 wild type; b, *S. sanguis* Challis DL125; c, *C. piscicola* LV17.TC1001; d, C2.NZ; e, TC3501; f, TC3008; g, TC3504.

ml, whereas chloramphenicol resistance of transformants increased from less than 1 to 64 µg per ml of agar. Transformants containing pNZ12d, designated as *C. piscicola* LV17.C2NZ, were used as the recipient strain for the second stage conjugation with pAMß1-containing strain TC1001.

In a preliminary experiment, the interaction between DNase I and protease in the presence of Ca^{2+} was determined to circumvent digestion of DNase I by protease in the DNase-treated control. The addition of 5 mM Ca^{2+} stabilized the activity of DNase I in the presence of trypsin and protease I, as shown in Table 4.3. However, Pronase E prepared in distilled water did not inactivate DNase I, regardless of added Ca^{2+}. This may be due to presence of Ca acetate in the enzyme preparation. Therefore, Pronase E was used for the second stage conjugation experiment to overcome the lethal effect of bacteriocin produced by the Bac^+ donor strain against the Bac^- recipient strain. However, 5 mM Ca^{2+} was added to the DNase I-treated mating mixture containing Pronase E to guard against possible protease inactivation of DNase I.

Results of the second stage conjugation between the pAMß1-containing Bac^+ Em^r Cm^s strain *C. piscicola* LV17.TC1001 as donor and the Bac^- Em^s Cm^r *C. piscicola* LV17.C2NZ as recipient are shown in Table 4.4. Conjugation efficiencies of filter mating were ten times greater than solid surface mating, and addition of 5 mM Ca^{2+} increased the efficiency of pAMß1 transfer 1.5x to 2.4x regardless of the mating method. Addition of bacteriocin produced by *C. piscicola* LV17 to the screening medium containing 10 µg Cm and 10 µg Em per ml reduced the conjugation efficiency by 5 to 50 times compared with the rate on the screening medium containing the antibiotics alone. Transconjugants on all of the screening media were randomly selected and checked for bacteriocin production. Transconjugants isolated from CmEm-containing screening media showed three plasmid profiles and two levels of bacteriocin activity: (a) only pAMß1 was transferred to the LV17.C2NZ recipient (Fig. 4.2-B, lane 1); or (b) pAMß1 was cotransferred with pCP40 (Fig. 4.2-B, lane 2) with partial bacteriocin activity of approximately 20% of wild type

Table 4.3. Effect of added calcium on the stability of DNase I in the presence of selected proteases

Enzyme Treatment	Calcium ions, 5 mM	DNase activity on DTA[a]
DNase I alone	added	+
	not added	+
DNase I + trypsin	added	+
	not added	-
DNase I + protease type 1	added	+
	not added	-
DNase I + pronase E	added	+
	not added	+

[a] DTA - DNase Test agar (Difco)

Table 4.4. Conjugation efficiencies between *C. piscicola* LV17.TC1001 and LV17.C2NZ

Treatment	Donor CFU/ml	Efficiency[a,b] CmEm	CmEmBac	Efficiency[c] of cotransfer
(1) Solid surface mating				
Pronase	1.8×10^{9}	1.0×10^{-5}	2.3×10^{-7}	2.2×10^{-2}
Pronase + Ca^{2+}	1.4×10^{9}	1.7×10^{-5}	2.6×10^{-7}	1.6×10^{-2}
Pronase + Ca^{2+} + DNase I	1.2×10^{9}	1.7×10^{-5}	1.7×10^{-7}	1.0×10^{-2}
(2) Filter mating				
Pronase	3.8×10^{8}	2.6×10^{-4}	1.7×10^{-5}	6.6×10^{-2}
Pronase + Ca^{2+}	4.0×10^{8}	6.2×10^{-4}	1.7×10^{-5}	2.8×10^{-2}
Pronase + Ca^{2+} + DNase I	7.1×10^{8}	4.8×10^{-4}	1.1×10^{-5}	2.2×10^{-2}

[a] CmEm = APT agar with 10 µg chloramphenicol and 10 µg erythromycin per ml of medium.
CmEmBac = CmEm with bacteriocin from *C. piscicola* LV17 (see Materials and Methods).

[b] Efficiency expressed as number of transconjugants per donor on each screening agar.

[c] Mobilizing efficiency of pAMß1 expressed as number of Bac^{+} transfers per transfer of pAMß1. All numerical values obtained are averages of two identical experiments.

activity (Fig. 4.3, zone f); or (c) pAMß1 was cotransferred with all three donor plasmids (Fig. 4.2-B, lane 3) with bacteriocin activity equivalent to the wild type strain (Fig. 4.3, zone e). Bac^p and Bac$^+$ transconjugants were 2 and 24% of the colonies tested, respectively. No transconjugants containing pCP49 alone were detected.

Transconjugants screened on CmEm-bacteriocin plates showed bacteriocin activity equivalent to the wild type strain. However, two types of plasmid profiles were detected: transfer of all three donor plasmids as described above (Fig. 4.2-C, lane 2); and transfer of pAMß1 with appearance of a new 89Mda plasmid (pCP89), which shows wild type Bac$^+$ activity (Fig. 4.2-C, lane 1, and Fig. 4.3, zone g). Mobilization efficiency of pAMß1 for the bacteriocin plasmids, expressed as the number of Bac plasmid transfers per pAMß1 transfer (Table 4.4) was 2 x 10^{-2} regardless of the mating method used. DNase I treatment did not affect the conjugation efficiency, as shown in Table 4.4. Mating mixtures of recipient cells and donor filtrate or donor cells and recipient filtrate did not give rise to transconjugants.

Results from experiments to check the immunity of each type of transconjugant are illustrated in Fig. 4.3 (panels B to D). Transconjugant TC3008, which received pCP40 and produces bacteriocin active against *C. divergens* LV13 and Bac$^-$ plasmidless mutant *C. piscicola* LV17.C2 (Fig. 4.3-A and B, zone f), showed immunity to the bacteriocin produced by itself, but it was still sensitive to bacteriocins produced by strains containing both plasmids, pCP40 and pCP49, or pCP89 (Fig. 4.3-C). However, the latter stains showed intact immunity to wild type strain *C. piscicola* LV17 (Fig. 4.3-D). Restriction digestion patterns of DNA's from each transconjugant are illustrated in Fig. 4.4. When subjected to digestion by *Bgl*II, the first-stage transconjugant TC1001 showed the presence of undigested pAMß1 and 13 digested fragments from 3 residential plasmids, pCP9, pCP40, and pCP49 (Fig. 4.4A, lane 4). Compared with the digestion pattern of pCP9 only (Fig. 4.4A, lane 2) and a mixture of pCP40 and pCP49 (Fig. 4.4A, lane 3), there was no change in molecular intactness. All the second stage transconjugants showed the presence

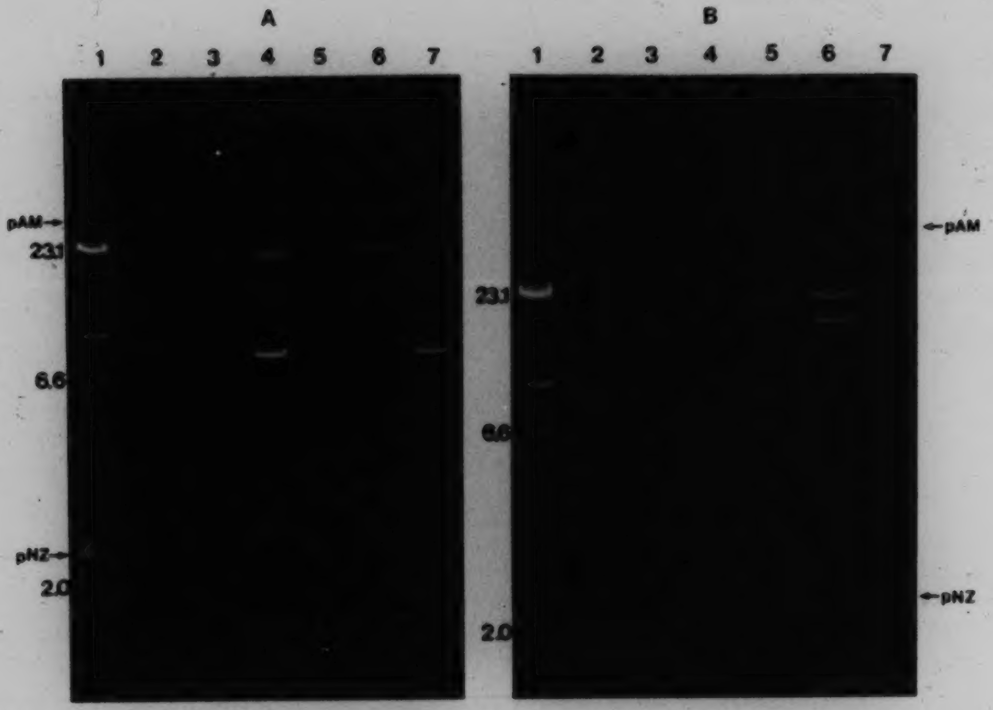

Fig. 4.4. ***BglII*** **digestion pattern of plasmids from transconjugants (panel A), cointegrate, and destabilization product (panel B).**

Lane 1 of each panel, mobility standard of *Hin*dIII digest of bacteriophage λ DNA; lane 2 of each panel, purified pCP9 DNA; lanes A-3 and B-7, mixture of pCP40 and pCP49 DNA isolated from gels; lane A-4, *C. piscicola* LV17.TC1001; lane A-5, TC3001; lane A-6, TC3008; lane A-7, TC3501; lane B-3, TC3008M5; lane B-4, pCP49 DNA isolated from gel; lane B-5, TC3504; lane B-6, TC3504M4. Undigested pAMβ1 and pNZ12d are marked with arrows. Gel electrophoresis shown in panel A was carried out at 5.0 V/cm, while gel electrophoresis shown in panel B was at 3.3 V/cm to differentiate larger DNA bands. Sizes of fragments of mobility standard are expressed in kilobases.

of pAMß1 (Fig. 4.4A, lanes 5 to 7, and Fig 4.4B, lane 5). The digestion pattern of TC3008 confirmed that only pCP40 was conjugally transferred with pAMß1 (Fig. 4.4A, lane 6), while that of TC3501 showed all residential plasmids of TC1001 were co-transferred with pAMß1 (Fig. 4.4A, lane 7). The restriction pattern of pCP89, a new large plasmid which appeared in TC3504, showed almost the same pattern as that of the mixture of pCP40 and pCP49 DNA (Fig. 4.4B, lanes 5 and 7, respectively), confirming that it is a co-integrate plasmid between pCP40 and pCP49. However, a 15.4 Kb fragment from pCP40 (Fig. 4.4B, lane 3) and a 25.5 Kb fragment from pCP49 (Fig. 4.4B, lane 4) were absent in the digestion pattern of co-integrate pCP89 and a new 11.2 Kb fragment had appeared. The 24.9 Kb DNA band of co-integrate digest seemed to be two overlapping fragments, judging from its intensity.

Destabilization of the antibiotic marker plasmids, pNZ12d and pAMß1, from transconjugant TC3008 by subculturing it without selective pressure of chloramphenicol and erythromycin resulted in loss of both plasmids in TC3008M5, which contains only pCP40 (Fig. 4.2-D, lane 3, and Fig. 4.4B, lane 3). However, destabilization of transconjugant TC3504 containing co-integrate pCP89 caused loss of pNZ12d and appearance of two new plasmids of 37 MDa and 51 MDa (Fig. 4.2-E). The *Bgl*II digestion pattern of these plasmids (Fig. 4.4B, lane 6) was the same as that of co-integrate pCP89 except that one of the 24.9 Kb fragments seemed to be resolved into 17.9 and 6.8 Kb fragments. However, bacteriocinogenicity and immunity of the strain TC3504M4, containing two resolved plasmids of pCP89, was not affected by the event.

4.4. Discussion

The nonconjugative plasmids, pCP40 and pCP49, associated with bacteriocin production and resistance in *C. piscicola* LV17, were successfully mobilized into the plasmidless (Bac^-) mutant *C. piscicola* LV17.C2. Self-transmissibility of bacteriocin plasmids has been reported for nisin (29), and bacteriocin plasmids of *Pediococcus acidilactici* (21), lactococci (18), and lactobacilli (17). These self-transmissible bacteriocin plasmids, with molecular weights ranging from 7.4 to 75 MDa, were conjugally transferred to heterospecies or Bac^- mutants of the producer strain with concomitant resumption of bacteriocin production. In our study, self-transmissibility of pCP40 and pCP49 between *C. piscicola* LV17 and its plasmidless mutant was not detected under the conditions of the experiment, which used pNZ12d to give the recipient strain a selection marker. Therefore it was interpreted that pCP40 and pCP49 are nonconjugative. However, this may be due to the lack of more efficient detection procedures (9, 16).

Because of the difficulty with self-transmission of pCP40 and pCP49, a two-stage conjugation experiment was devised utilizing the broad host range and mobilizing ability of pAMß1 (8). Conjugal transfer of pAMß1 from *S. sanguis* to *C. piscicola* LV17 containing its three resident plasmids occurred with an efficiency of 10^{-6} transconjugants per donor. This was 10- to 100-fold greater than the transfer efficiency observed between *E. faecalis* JH2-2 and *Lactobacillus plantarum* (25), but 1000-fold less than the transfer efficiency to *L. lactis* subsp. *lactis* or *Lactobacillus acidophilus* ADH (14). The second stage conjugation between the pAMß1-containing strain LV17.TC1001 and chloramphenicol resistant Bac^- mutant LV17.C2NZ resulted in 10^{-4} Em^r transconjugants per donor with filter mating and 10^{-5} Em^r transconjugants per donor with solid surface mating, showing that pAMß1 residing in *Carnobacterium* sp. was intact and retained its conjugative ability for second stage intrageneric transfer.

Co-transfer or mobilization of the bacteriocin plasmids occurred in three ways: (a) co-transfer of all resident plasmids in *C. piscicola* LV17 with resumption of wild-type Bac^+ activity in the recipient; (b) co-transfer of pCP40 with partial resumption of bacteriocin activity (Bac^p); and (c) appearance of an 89MDa cointegrate of pCP40 and pCP49 with wild-type Bac^+ activity in the recipient. The mechanism of mobilization of the nonconjugative plasmids by pAMß1 is not clear. Three mechanisms for mobilization are suggested with pAMß1, namely: (a) co-integrate formation as in pAMß1::pOD1 formation in the mobilization of nonconjugative pOD1 from *Bacillus subtilis* to *Clostridium acetobutylicum* (19); (b) mobilization without integration with pAMß1; and (c) a combination of these processes as reported for mobilization of the proteinase plasmids from *L. lactis* subsp. *lactis* UC317 to *L. lactis* subsp. *cremoris* UC205 (9). In our study, cointegration of the Bac plasmids with pAMß1 was not detected, hence mobilization without integration is assumed. However, it is not clear whether a transit cointegrate was formed between the Bac plasmids and pAMß1, similar to the pVA797::pVA838 transit cointegrate reported in *E. faecalis* (27).

The nature of the cointegrate of pCP40 and pCP49 formed during conjugation indicates the possibility that a transposon-like element is present in the Bac plasmids (7, 8), as observed in 104 Kb co-integrate plasmid pPW2 by Anderson and McKay (3) during conjugal transfer of cell aggregation phenomena in *L. lactis* ML3. Digestion of the cointegrate with *Bgl*II produced two new junction fragments replacing two fragments from pCP40 and pCP49. Subculturing of the cointegrate-containing transconjugants resulted in the resolution of pCP89 into two new plasmids of 37 and 51 MDa. Because similar plasmids were found in the regenerated cells of *C. piscicola* LV17 during protoplast-induced curing in our earlier study (2), detection of pCP40 and pCP49 cointegrates was expected. However, it is not certain whether conjugation or mobilization by pAMß1 stimulated the cointegrate formation between the two Bac plasmids of *C. piscicola* LV17.

Incorporation of chloramphenicol resistance into the Bac$^-$ plasmidless mutant of *C. piscicola* LV17 by electroporation of pNZ12 provided an efficient system for screening transconjugants which were otherwise indiscernible from the Bac$^+$ donor strain. Furthermore the instability of pNZ12 could be exploited to remove the Cm^r selection plasmid from transconjugants by successive subculturing without Cm selective pressure. The reason for the disruption of the kanamycin resistance region identifiable with *Bgl*II is not clear. Because the kanamycin resistance of pNZ12 was not disrupted by electrotransformation in other carnobacteria, *C. piscicola* UAL26 and *C. divergens* LV13 (unpublished data), the molecular change in the kanamycin resistance-encoding region in *C. piscicola* LV17 may be due to a strain-specific restriction-modification system affecting this region or physical forces during electrotransformation. The presence of transconjugants containing pAMß1 and pNZ12d showed that conjugal transfer occurred from the 4-plasmid containing donor to the pNZ12d-containing recipient, and indicated that pNZ12 is not conjugative. The modified pNZ12, pAMß1 and the resident plasmids of *C. piscicola* LV17 are compatible.

Mobilization of bacteriocin plasmids from *C. piscicola* LV17 by pAMß1 and subsequent removal of pAMß1 by destabilization can be exploited as a method for transferring nonconjugative plasmids to a target microorganism, leading to improvement of starter cultures for use in food technology. This gives the potential to transfer desired bacteriocin genes between strains without modifying the plasmids. Further studies on improved cultures with broader antimicrobial spectra and bacteriocin compatibility are in progress. This could be especially useful for competitive growth of starter bacteria in mixed culture (12).

4.5. References

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5. Mobilization and Location of the Genetic Determinant of Chloramphenicol Resistance from *Lactobacillus plantarum* caTC2R[1]

5.1. Introduction

Lactobacillus plantarum caTC2R isolated from raw, ground pork showed chloramphenicol resistance dependent upon chloramphenicol acetyl transferase (CAT) activity, and mediated by an 8.5 Kb plasmid (9). Lack of an appropriate marker or genetic delivery system has been a major problem for strain improvement of meat starter cultures by genetic technology (4, 11), hence the possible use of this 8.5 Kb plasmid for a chloramphenicol resistance marker or as vector DNA is of interest. Further study of this plasmid in *L. plantarum* caTC2R was hindered by the presence of two other residential plasmids. Attempts to separate the 8.5 Kb plasmid by electrotransformation of a mixture of all three plasmids into a plasmidless strain of *Carnobacterium piscicola* LV17 (2) with selection for chloramphenicol resistance was unsuccessful. Conjugal transfer of the 8.5 Kb plasmid was also unsuccessful. To circumvent this problem, the conjugative broad host-range plasmid pAMß1 (5) with macrolide-lincosamide-streptogramin B (MLS) resistance was selected as a mobilizing agent (6). pAMß1 has a broad host range in lactic acid bacteria and it has been used successfully to mobilize nonconjugative plasmids in various species (6), including proteinase plasmids in *Lactococcus lactis* subsp. *lactis* UC317 (7) and bacteriocin plasmids in *C. piscicola* LV17 (see Chapter 4).

This study reports the successful mobilization of the 8.5 Kb chloramphenicol resistance plasmid (pCaT) from *L. plantarum* caTC2R by pAMß1-associated mobilization technique, followed by intensive restriction mapping of the plasmid and location of the chloramphenicol resistance gene. Expression of chloramphenicol resistance in other species is also reported.

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[1] A version of this chapter will be published by Ahn, C., D. Collins-Thompson, and M.E. Stiles in Applied and Environmental Microbiology. The information in this chapter excludes the contribution to be made by Dr. D. Collins-Thompson, Department of Environmental Biology, University of Guelph.

5.2. Materials and Methods

Bacterial strains and culture media

Bacterial strains used in this study are listed in Table 5.1. *L. plantarum* caTC2R was isolated as a result of screening lactic acid bacteria from meats for chloramphenicol (Cm) resistance (9). *Streptococcus sanguis* Challis DL125 containing the erythromycin (Em) resistant, conjugative plasmid pAMß1 was used as the donor for this plasmid. A plasmidless strain of *Carnobacterium piscicola* LV17.C2 (2) was used as a recipient of pAMß1 and (or) pCaT, the 8.5 Kb Cm^r plasmid from *L. plantarum* caTC2R. *Carnobacterium divergens* LV13 was used as the indicator strain for bacteriocinogenic (Bac$^+$) strains. *E. coli* BHB 2600 containing pMG36e (19) was obtained from G. Venema (Department of Genetics, University of Groningen, Groningen, The Netherlands). Stock cultures were stored in Cooked Meat medium (Difco Laboratories Inc., Detroit, MI) at 4°C, and subcultured in APT broth (Difco) at least twice before use in experiments. LB broth (14) was used for growing the *E. coli* strains. Where appropriate, antibiotics were added to the APT broth to ensure antibiotic resistance of the strains.

Conjugation experiments

The experimental scheme for two stage conjugation to mobilize the 8.5 Kb plasmid by pAMß1 is illustrated in Fig. 5.1. The procedures were outlined in Chapter 4 (p. 59), except that a 0.22 µm Nucleopore filter membrane was used in place of the Millipore filter, and proteinase was not added to the conjugation mixture because there was no antagonism between donor and recipient cells. Conjugation mixtures were incubated at 30°C for 18 h under aerobic conditions. Transconjugants were screened by fermentation patterns for the appropriate carbohydrates and by plasmid profiles.

Table 5.1. Bacterial strains.

Strain	Phenotype (plasmid)	Source
Streptococcus sanguis Challis DL125	Em^r (pAMß1)	10
Lactobacillus plantarum		
caTC2R	Cm^r mal^-	9
caTC2R.pAM	Cm^r Em^r mal^-	This study
Carnobacterium piscicola		
LV17.C2	Cm^s mal^+	2
LV17.C2DP	Cm^r Em^r mal^+	This study
LV17.C2D	Cm^r mal^+	This study
UAL26	Cm^s mal^+	1
UAL26D	Cm^r mal^+	This study
Carnobacterium divergens		
LV13	Cm^s mal^+	16
LV13D	Cm^r mal^+	This study
Escherichia coli BHB 2600	Em^r (pMG36e)	19

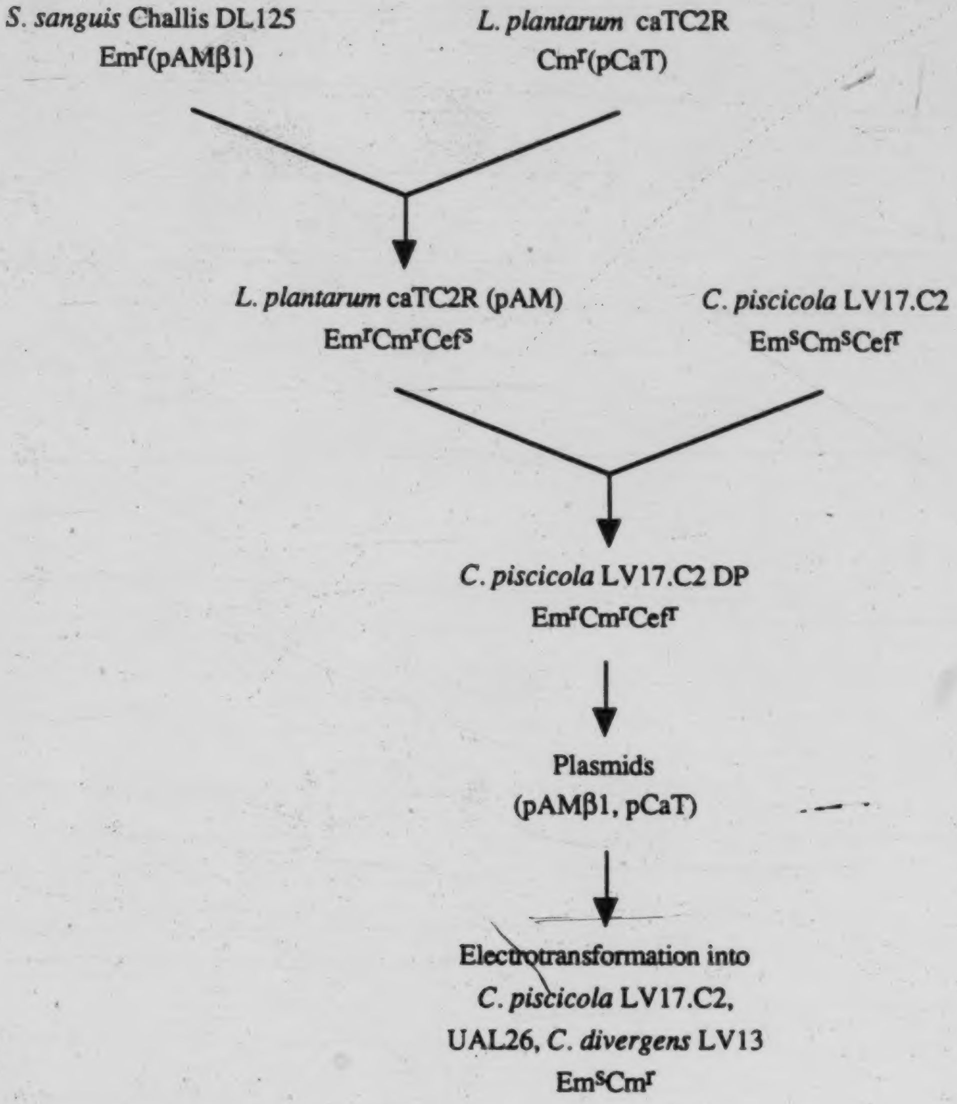
S. sanguis Challis DL125
Em^{r}(pAMβ1)
L. plantarum caTC2R
Cm^{r}(pCaT)
L. plantarum caTC2R (pAM)
Em^{r}Cm^{r}Cef^{s}
C. piscicola LV17.C2
Em^{s}Cm^{s}Cef^{r}
C. piscicola LV17.C2 DP
Em^{r}Cm^{r}Cef^{r}
Plasmids
(pAMβ1, pCaT)
Electrotransformation into
C. piscicola LV17.C2,
UAL26, *C. divergens* LV13
Em^{s}Cm^{r}

Fig. 5.1. Experimental scheme for mobilization of 8.5 Kb Cm^{r} plasmid pCAT by pAMß1 and separation of pCAT from pAMß1 by electrotransformation.

Em, erythromycin; Cm, chloramphenicol; cef, cephaloridine;
r, resistant to the antibiotic; s, sensitive to the antibiotic.

Plasmid extraction

The method described by Ahn and Stiles (2) was used for miniscale preparation of plasmids. Large scale extraction of plasmid DNA was done by 100 x scale-up of the miniprep technique. If necessary, the plasmid DNA was further purified by CsCl density gradient centrifugation (14). Gel electrophoresis was done on 0.8% agarose gel (Pharmacia LKB Biotechnology 5-75182, Uppsala, Sweden) in TAE buffer (40 mM Tris acetate, 1 mM EDTA) at 5V/cm for 3 h.

Separation of pCaT and expression in other strains

Separation of the plasmids was done by electrotransformation of the plasmid DNA extracted from the second-stage transconjugant containing the pAMβ1 and pCaT plasmids into *C. piscicola* LV17.C2 and screening for Em^s and Cm^r electrotransformants. Electrotransformation was done with a Gene Pulser^{TM} (Bio-Rad Laboratories, Richmond, CA) according to the manufacturer's protocol in HEPES-buffered sucrose electroporation buffer (HEB; 272 mM sucrose, 1 mM $MgCl_2$, 7 mM HEPES pH 7.4) with a single pulse at 25 μFD and 200 ohms resistance at 12.5 KV/cm. The 8.5 Kb (Cm^r) plasmid from the transformant was further purified by CsCl density gradient ultracentrifugation and electrotransformed into *C. piscicola* UAL26, *C. divergens* LV13, and *Leuconostoc mesenteroides* UAL 60. Cm^r transformants of each strain were checked for their plasmid profiles and minimum inhibitory concentration (MIC) of chloramphenicol in APT agar.

Restriction analysis

Purified 8.5 Kb plasmid DNA was digested with selected restriction enzymes (Boehringer Mannheim Canada, Laval, Quebec) using procedures specified by the supplier and mapped by procedures described by Sambrook *et al.* (14). Calibration of fragment sizes was done by multiple regression analysis (13) using the *Hin*dIII digest of bacteriophage λ DNA (Boehringer Mannheim) as mobility standards.

Cloning

Cloning of the DNA fragment encoding chloramphenicol resistance in the 8.5 Kb plasmid was done by the procedure described by Sambrook *et al.* (14). The 8.5 Kb plasmid cut by various sets of restriction enzymes was ligated with pMG36e vector DNA which was cut by the same set of restriction enzymes. T4 DNA ligase was obtained from BRL (Bethesda Research Laboratories, Life Technologies, Inc., Gaithersburg, MD) and used as recommended by the supplier. However, ligation buffer without ethylene glycol was used for the ligation reaction (14). The ligation mixture was incubated at 16°C for 6 h, and the cloned DNA was transformed into plasmidless *C. piscicola* strains LV17.C2 or UAL26 by electrotransformation as described earlier. Transformants were screened on the APT agar plates containing 8 µg of chloramphenicol and 10 µg of erythromycin per ml of agar.

5.3. Results

Results of the first-stage conjugation to transfer pAMß1 from *S. sanguis* Challis DL125 to *L. plantarum* caTC2R are shown in Table 5.2. The conjugation efficiency expressed as the number of Cm^r Em^r transconjugants per donor cell was 4×10^{-5}, and it was not affected to any marked extent by treatment with DNase I. Control conjugation experiments with filtrates of donor or recipient cells with the respective cells did not reveal any transconjugants on the Cm-Em screening plates. The plasmid profile of the transconjugant designated as *L. plantarum* caTC2R.pAM showed the presence of 26.5 Kb pAMß1 and the three residential plasmids of the *L. plantarum* strain (Fig. 5.2, lanes 2 to 4). All transconjugants grew but failed to ferment maltose, confirming that the origin of the transconjugants is *L. plantarum* caTC2R.

The second-stage conjugation between *L. plantarum* caTC2R.pAM and *C. piscicola* LV17.C2 occurred with an efficiency of 2.01×10^{-6} transconjugants per donor (Table 5.2).

Table 5.2. Efficiency of conjugal transfer of pAMß1 from *S. sanguis* Challis DL125 to *L. plantarum* caTC2R and efficiency of co-transfer of pAMß1 and pCaT from *L. plantarum* caTC2R.pAM to *C. piscicola* LV17.C2.

Experiment	Donor	Recipient	DNase I[a] treatment	CFU/ml[b] Donor	Efficiency (transconjugants/ donor)
1st stage conjugation	*S. sanguis* Challis DL125	*L. plantarum* caTC2R	not-treated	1.62×10^{9}	4.12×10^{-5}
			treated	1.52×10^{9}	2.41×10^{-5}
2nd stage conjugation	*L. plantarum* caTC2R.pAM	*C. piscicola* LV17.C2	not-treated	1.35×10^{8}	2.01×10^{-6}
			treated	2.64×10^{8}	1.93×10^{-6}

[a] 1 mg/ml of DNase I was added to the conjugation mixture.

[b] Colony forming units per ml.

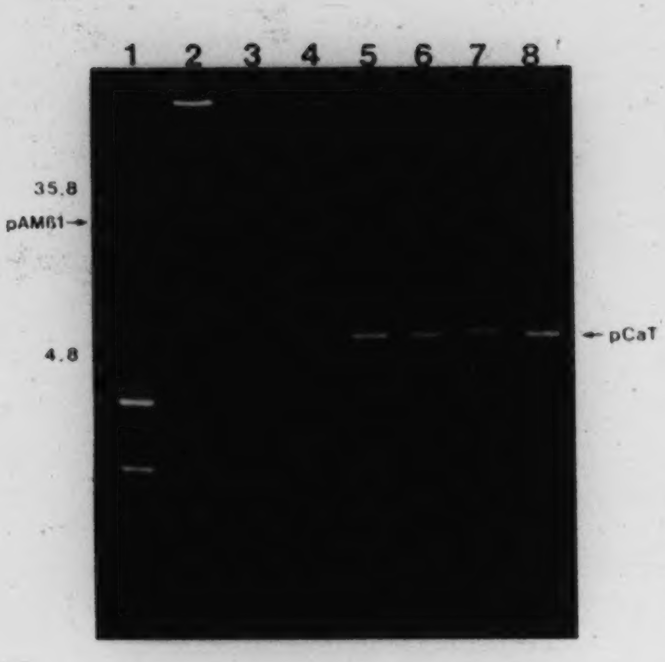

Fig. 5.2. Plasmid profiles of transconjugants and electrotransformants.

Lane 1, mobility standard *E. coli* V517; lane 2, *L. plantarum* caTC2R; lane 3, *S. sanguis* Challis DL125; lane 4, *L. plantarum* caTC2R.pAM; lane 5, *C. piscicola* LV17.C2DP; lane 6, *C. piscicola* LV17.C2D; lane 7, *C. piscicola* UAL26D; and lane 8, *C. divergens* LV13D. Molecular weights of mobility standard are expressed as megadaltons (MDa), and location of pAMß1 and pCaT is marked as pAM and pCaT, respectively.

Plasmid DNA profiles of the transconjugant cells confirmed that transfer of Cm resistance was due to co-transfer of the 8.5 Kb plasmid with pAMß1 (Fig. 5.2, lane 5). Carbohydrate fermentation patterns of the transconjugants, including maltose, sucrose, and lactose in the presence of chloramphenicol, cephaloridine and erythromycin as selective agents identified the origin of the transconjugants as *C piscicola* LV17.C2 (1, 2). The efficiency of conjugation in the presence of DNase I was not changed (Table 5.2), and controls with cell filtrates did not show any transconjugants on the screening media.

The *C. piscicola* LV17.C2 transconjugant containing pAMß1 and the 8.5 Kb Cm^r plasmid was subcultured in APT broth without addition of Em for 50 generations but Em^s strains were not detected. However, when plasmid DNA from this strain was electrotransformed into plasmidless *C. piscicola* LV17.C2 (time constant 2.2 ms at 12.5 KV/cm, 25 µFD and 200 ohms), all electrotransformants that appeared on the chloramphenicol-containing screening media were erythromycin-sensitive. Plasmid profiles of the transformants confirmed that only the Cm^r plasmid had been transformed (Fig. 5.2, lane 6). The 8.5 Kb plasmid in these transformants was prepared by large scale extraction and purified by CsCl gradient ultracentrifugation. The purified plasmid was again electrotransformed into *C. piscicola* LV17.C2 as an efficiency control, and into *C. piscicola* UAL26, *C. divergens* LV13, and *Leuc. mesenteroides* UAL60. The efficiency of these transformations under the same conditions of electrotransformation are shown in Table 5.3. High efficiencies of 10^4 to 10^5 transformants were achieved per µg of DNA for the strains of *C. piscicola* but low frequencies of 10^1/µg DNA were achieved for other organisms, and no transformants were detected for *Leuc. mesenteroides* UAL 60. Plasmid profiles of the transformants showed that the 8.5 Kb plasmid is present intact in *Carnobacterium* (Fig. 5.2, lanes 7 and 8). The presence of the 8.5 Kb plasmid increased the Cm^r of the transformants to 32 to 64 µg/ml when tested on APT agar.

Table 5.3. Efficiency of electrotransformation of pCaT into various strains of *Carnobacterium* and minimum inhibitory concentration (MIC) of chloramphenicol in each transformant.

Strain	Time Constant[a] (ms)	Efficiency (transformants/ µg DNA)	MIC (µg/ml of agar)
C. piscicola LV17.C2	2.3	5.91×10^4	64
C. piscicola UAL26	2.2	2.84×10^5	32
C. divergens LV13	2.0	1.09×10^1	64

[a] Time constant for electrotransformation expressed in milliseconds (ms).

The 8.5 Kb plasmid was mapped with 12 kinds of restriction enzymes. As shown in Fig. 5.3, the plasmid has unique restriction sites for *Ava*I, *Eco*RI, *Eco*RV, *Hin*dII, *Hin*dIII, *Hpa*II, *Pst*I, *Sal*I, and *Xba*I, and two restriction sites for each of *Acc*I, *Bgl*II, and *Sph*I. The *Acc*I-*Hin*dII sites at the 6.9 Kb point, and *Sal*I-*Bgl*II sites at the 6.6 Kb point from *Hin*dIII site are too close to map their correct positions.

Results of the cloning experiments for the DNA fragment encoding chloramphenicol resistance are shown in Fig. 5.4 and 5.5. The 5.9 Kb fragment of *Pst*I - *Hin*dIII (Fig. 5.4A, lane 2), 6.6 Kb fragment of *Sal*I - *Hin*dIII (Fig. 5.4A, lane 4), 4.0 Kb fragment of *Pst*I - *Sal*I (Fig. 5.4A, lane 6), 6.4 Kb fragment of *Sph*I (Fig. 5.4B, lane 2), and 5.7 Kb fragment of *Eco*RI - *Eco*RV (Fig. 5.4C, lane 3) were cloned into pMG36e, rendering transformants resistant to chloramphenicol up to 32 or 64 µg/ml. The 2.6 Kb *Eco*RV - *Sal*I region of the 8.5 Kb plasmid that is responsible for chloramphenicol resistance is illustrated in Fig. 5.5.

5.4. Discussion

The separation of the nonconjugative 8.5 Kb plasmid associated with chloramphenicol resistance in *L. plantarum* caTC2R (9) from the other two residential plasmids was achieved by introduction of the conjugative plasmid pAMß1 into *L. plantarum* caTC2R in the first stage conjugation, and by mobilization of the 8.5 Kb plasmid, together with pAMß1 in the second stage conjugation into the plasmidless strain of *C. piscicola* LV17.C2. Expression of chloramphenicol resistance in a heterospecies such as *Carnobacterium* proved that the 8.5 Kb plasmid mediates chloramphenicol resistance and excluded the possibility of a concerted mode of action with the chromosome or other residential plasmids in *L. plantarum*. Furthermore, separation of the 8.5 Kb plasmid from pAMß1 in transconjugants was easily achieved by separative electrotransformation, proving the applicability of the technique to remove pAMß1 when used as a mobilizing agent for nonconjugative plasmid far smaller than itself.

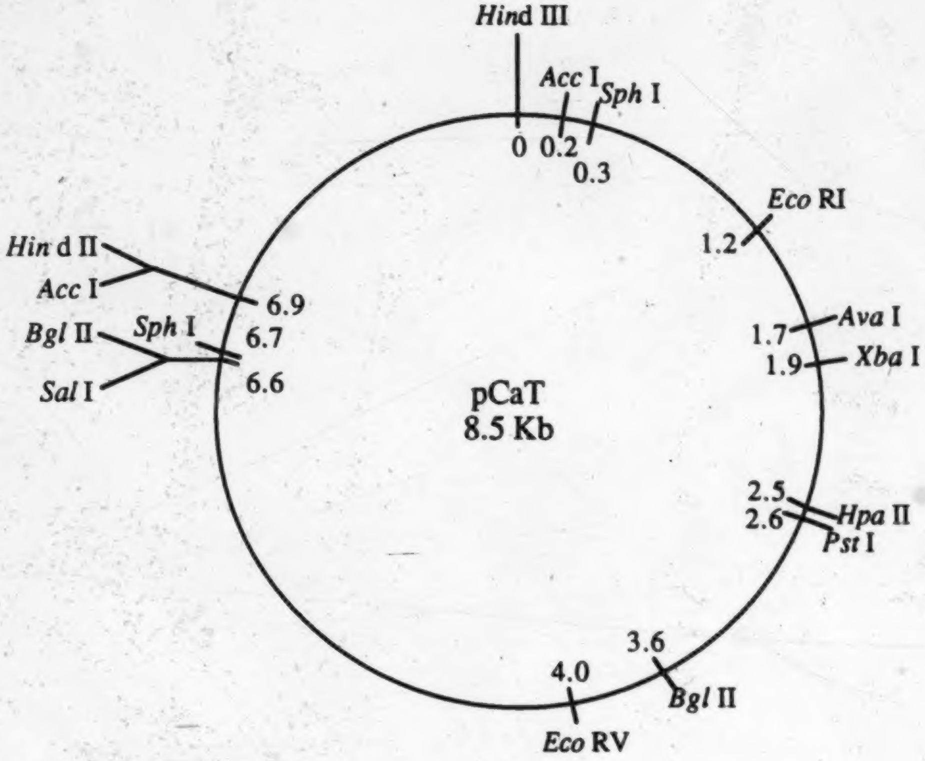
Hind III
Acc I
Sph I
0
0.2
0.3
Eco RI
1.2
1.7
Ava I
1.9
Xba I
2.5
2.6
Hpa II
Pst I
3.6
Bgl II
4.0
Eco RV
6.6
6.7
6.9
Hin d II
Acc I
Bgl II
Sph I
Sal I
pCaT
8.5 Kb

Fig. 5.3. Restriction map of 8.5 Kb Cm^r plasmid pCaT.

*Hind*III site is arbitrarily assigned as 0.

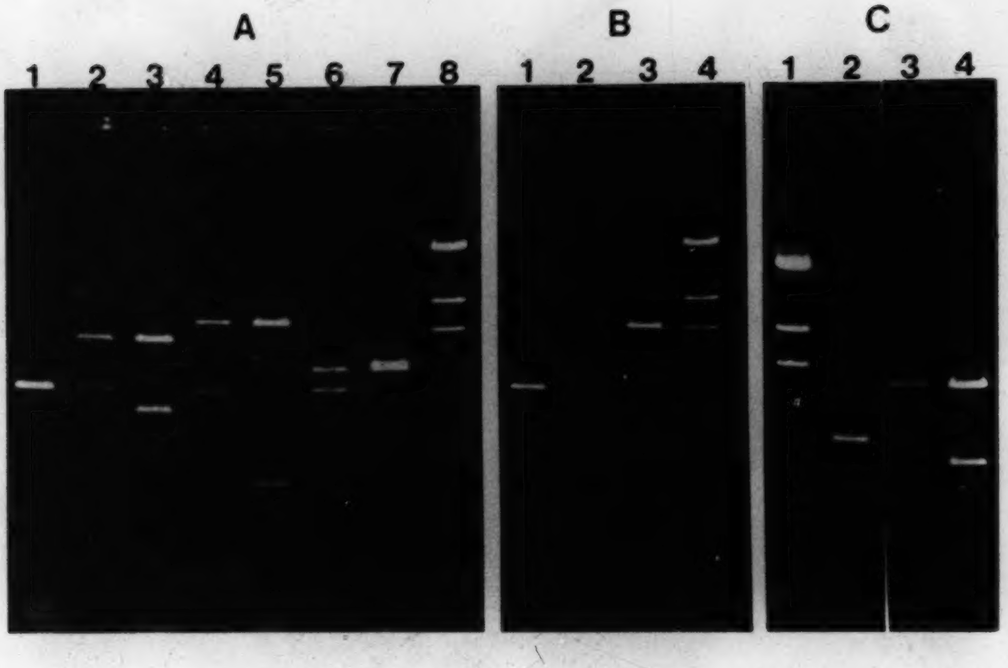

Fig. 5.4. Restriction analysis of cloned fragments from pCaT.

Panel A: lanes 1 to 3, *Pst*I - *Hin*dIII digest of pMG36e, pMG36e V261, and pCaT; lanes 4 and 5, *Sal*I - *Hin*dIII digest of pMG36e W261 and pCaT; lanes 6 and 7, *Pst*I - *Sal*I digest of pMG36e X261 and pCaT;

Panel B: lanes 1 to 3, *Sph*I digest of pMG36e, pMG36e Rd261, and pCaT;

Panel C: lanes 2 to 3, *Eco*RI - *Eco*RV digest of pMG36e, pMG36e N263, and pCaT.

Mobility standard of *Hin*dIII digest of bacteriophage λ DNA is shown in lanes A-8, B-4, and C-1.

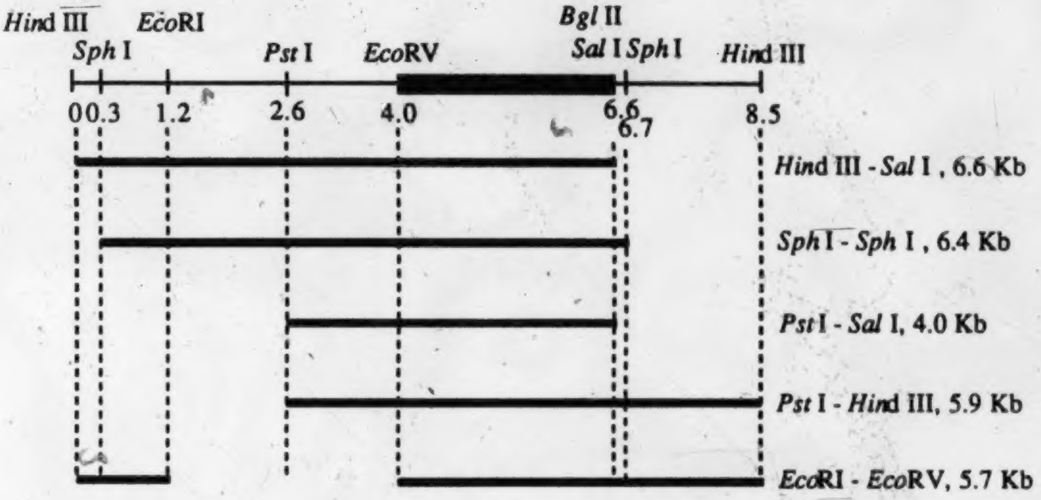
Hind III
EcoRI
Sph I
Pst I
EcoRV
Bgl II
Sal I
Sph I
Hind III
0
0.3
1.2
2.6
4.0
6.6
6.7
8.5
Hind III - Sal I , 6.6 Kb
Sph I - Sph I , 6.4 Kb
Pst I - Sal I, 4.0 Kb
Pst I - Hind III, 5.9 Kb
EcoRI - EcoRV, 5.7 Kb

Fig. 5.5. Summary of cloning experiments and location of chloramphenicol acetyl transferase (CAT) gene in pCaT.

Each fragment of pCaT cloned into pMG36e is shown as a thick line, and the 2.6 Kb *Eco*RV-*Sal* I region which contains CAT gene is illustrated as a heavy black bar.

The conjugal transfer efficiency of pAMß1 from *S. sanguis* Challis DL125 to *L. plantarum* caTC2R was similar to those reported by West and Warren (20) between *S. lactis* 712 lac$^-$ (pAMß1) and *L. plantarum* 340, 352 and 1752, and higher than those reported by Sasaki *et al.* (15) between *S. faecalis* JH2-2 (pAMß1) and *L. plantarum* JCM 1149 on a millipore filter membrane (0.45 µm). However, Sasaki *et al.* (15) did not detect any transconjugants when they used a Nucleopore filter membrane (0.4 µM). They attributed this to the structural characteristics of the membrane. Unfortunately, there was no data for a 0.22 µm Nucleopore filter membrane for *S. sanguis* Challis as donor. The successful conjugation in our experiments with the 0.22 µm Nucleopore filter membrane is either due to the strain-specific characteristics or to procedural differences.

Intergeneric transfer of pAMß1 from the first stage transconjugant *L. plantarum* caTC2R (pAMß1) to *Carnobacterium* in the second stage conjugation showed intactness of pAMß1 conjugativity (tra region of the plasmid) even after the first stage conjugation. West and Warner (20) also reported conservation of conjugal capability of pAMß1 when *L. plantarum* was used as the first host. However, the mechanism of cotransfer of 8.5 Kb plasmid with pAMß1 is uncertain because cointegrate formation, as reported by Oultram *et al.* (12) or cotransfer of residential plasmids other than the target plasmid, reported by Ahn and Stiles (Chapter 4) for bacteriocin plasmids of *Carnobacterium*, was not observed.

Results of cloning experiments defined a 2.6 Kb *Eco*RV - *Sal*I region of pCaT as responsible for chloramphenicol resistance. For the Gram-negative chloramphenicol acetyl transferase (CAT) gene, Alton and Vapnek (3) reported a 1,102 bp region of Tn9 which is responsible for chloramphenicol resistance in *E. coli*. The size of the structural gene itself was deduced as 657 bp from the primary structure data of the type I CAT enzyme (17). For the Gram-positive CAT gene, a staphylococcal chloramphenicol resistance plasmid pC194 was completely sequenced, and a 1,035 bp region was defined which was responsible for the structural gene, promoter and regulatory element for expression of chloramphenicol resistance (8). Compared with the data for other Gram-positive and

Gram-negative CAT genes, the 2.6 Kb region of pCaT needs further study to narrow down the region of the chloramphenicol resistance gene. As suggested by Smith and Burns (18), DNA homology studies with other Gram-positive and Gram-negative CAT genes would be a good way to trace the origin of chloramphenicol resistance in *L. plantarum* caTC2R.

The high transformation efficiency and intact expression of pCaT in *Carnobacterium* and the presence of several unique restriction sites create a good opportunity to use this plasmid in genetic studies of *Carnobacterium* species in two respects: using chloramphenicol resistance as a genetic marker in mixed culture studies, or developing the plasmid as vector DNA. To extend these possibilities, studies on expression of the plasmid in various Gram-positive strains and cloning of heterologous genes into the plasmid are being carried out in our laboratory.

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6. Cloning of a 9.6 Kb *Pst*I Fragment Encoding Bacteriocin Production and Immunity from a Bacteriocin Plasmid of *Carnobacterium piscicola* LV17

6.1. Introduction

Many of the lactic acid bacteria associated with dairy and meat products have been reported to produce bacteriocins, a class of bactericidal proteins that are generally active against closely associated strains of bacteria (10). In some instances these bacteriocins are active against a broader spectrum of bacteria, including spoilage and potentially pathogenic bacteria in food (5, 7). The potential to use these bacteria or their bacteriocins in food preservation is increasing as information on the nature of the bacteriocins and the genetics of bacteriocin production is compiled. Nisin produced by *Lactococcus lactis* subsp. *lactis* is being intensively studied. The molecular structure of the nisin peptide and its encoding gene have been reported (3, 6, 9). For other lactococcal bacteriocins, Scherwitz-Harmon and McKay (13) cloned an 18.4 Kb *Xho*I fragment that encodes bacteriocin production in *Lactococcus lactis* subsp. *diacetylactis* WM_4, and Van Belkum *et al.* (15) reported cloning of a 1.8 Kb *Sca*I-*Cla*I fragment and a 1.3 Kb *Sca*I-*Hin*dIII fragment encoding two different bacteriocins in a plasmid from *L. lactis* subsp. *cremoris* 984.

Carnobacterium piscicola isolated from vacuum-packaged meats (14) is a lactic acid bacterium that is closely related to, but separated from lactobacilli by several chemotaxonomic differences (4) including failure to grow on acetate agar at pH 5.6. *C. piscicola* LV17 produces a bacteriocin that is active against closely related strains of lactic acid bacteria and some strains of *Enterococcus* spp. and *Listeria monocytogenes* (2). Bacteriocin production is mediated by two plasmids, 49 and 40 MDa, which are responsible for 80% and 20% of apparent bacteriocin production, respectively (2). Characterization of the genetic determinant for bacteriocin production was hindered by the lack of an appropriate host-vector system for carnobacteria. Most Gram-positive vectors showed low transformation efficiency or molecular changes when used in *Carnobacterium*

(Ahn & Stiles, submitted for publication). An 8.5 Kb plasmid mediating chloramphenicol resistance (pCaT) from *Lactobacillus plantarum* was studied (see Chapter 5), and selected as a potential vector for *Carnobacterium*.

This study reports the cloning of a 9.6 Kb *Pst*I fragment encoding bacteriocin production and immunity from a 49 MDa plasmid (pCP49) responsible for 80% of bacteriocin activity in *C. piscicola* LV17, using a chloramphenicol resistance plasmid (pCaT) as a vector.

6.2. Materials and Methods

Bacterial strains and culture media

The bacterial strains used for the cloning studies are listed in Table 6.1. *C. piscicola* LV17 and *C. divergens* LV13 were obtained from B.G. Shaw (Institute of Food Research, Langford, Bristol, UK). *C. piscicola* LV17.C2D containing pCaT was used as a source microorganism for the plasmid pCaT. An *E. coli* strain containing the erythromycin resistance-encoding vector pMG36e (17) was obtained from G.Venema (Department of Genetics, University of Groningen, Haren, The Netherlands). Stock cultures were stored in Cooked Meat medium (Difco Laboratories Inc., Detroit, MI) at 4°C, and subcultured at least twice in APT broth at 25°C before use in experiments. *E. coli* strains were grown in LB broth (12) at 37°C on a rotary shaker.

Detection of bacteriocin activity

Production of bacteriocin by *C. piscicola* LV17 was detected by deferred antagonism and spot-on-lawn techniques described by Ahn and Stiles (1), with *C. divergens* LV13 as the indicator strain for general study of the inhibitory capacity of clones. Other indicator strains used to confirm activity of clones are listed in Table 6.3 (page 110).

Table 6.1. Bacterial strains and plasmids.

Strain	Plasmid(s)[a]	References/Sources
Carnobacterium piscicola		
LV17.C33	pCP49, pCP9	2
LV17.C99	pCP49	This study
LV17.C2	Plasmidless	2
LV17.C2D	pCaT	Chapter 5
LV17.C2NZ	pNZ12d	Chapter 4
UAL26	Plasmidless	1
LV17.TC3008M5	pCP40	Chapter 4
Carnobacterium divergens		
LV13	2.3 MDa plasmid	14
Escherichia coli		
BHB2600	pMG36e	17

[a] pNZ12d, pNZ12 without *Bgl*II-sensitive site.

Plasmid isolation and restriction enzyme digestion.

Miniprep isolation of plasmid DNA was done by methods described by Ahn and Stiles (2). Large scale extractions were done by 100 x scale-up of the miniprep technique, and plasmid DNA was purified by CsCl density gradient ultracentrifugation (12). Restriction enzymes were purchased from Boehringer Mannheim Canada (Laval, Quebec, Canada) and used according to the procedures specified by the supplier. Gel electrophoresis was done on 0.7% agarose gel (Pharmacia LKB Biotechnology, 5-75182 Uppsala, Sweden) in TAE buffer (40 mM Tris acetate, 1 mM EDTA) at 5 V/cm. Fragment size of DNA was determined by multiple regression analysis (11) with the *Hin*dIII digest of bacteriophage λ DNA (Boehringer Mannheim Canada) as mobility standards.

Protoplast-induced curing for *Carnobacterium piscicola* mutant containing pCP49

C. piscicola LV17 strain 33 which contains pCP49 and another cryptic 9.5 MDa plasmid (pCP9) was cured of pCP9 by protoplast-induced curing technique (2). Colonies growing on the regeneration medium were randomly picked and checked for plasmids. Colonies that lost pCP9 but retained pCP49 were further characterized by their carbohydrate fermentation pattern and restriction analysis of the plasmid DNA. The resultant mutant was used as the source of plasmid DNA for the cloning experiments. The differences between bacteriocins encoded by pCP49 and a 40 MDa plasmid (pCP40), which is responsible for 20% of bacteriocin activity in *C. piscicola,* were shown by cross-activity and immunity tests between them.

Molecular cloning

The procedures described by Sambrook *et al.* (12) were used. Ligation was done with T4 DNA ligase from BRL (Bethesda Research Laboratories, Life Technologies, Inc., Gaithersburg, MD) in ligation buffer without ethylene glycol (12). Vector and passenger DNA was cut with the same set of restriction enzymes and fragments were purified with phenol and chloroform, precipitated and resuspended in TE buffer (10 mM Tris•Cl, 1 mM

EDTA, pH 7.4) at a concentration of about 100 µg DNA/ml. Mixtures of purified digests of vector (pCaT or pMG36e) and passenger DNA were treated with T4 DNA ligase for 6 h at 16°C. The ligated DNA was electrotransformed into the plasmidless Bac⁻ strain of *C. piscicola* LV17.C2 with a Gene Pulser™ (Bio-Rad Laboratories, Richmond, CA) using the method recommended by the supplier and HEPES-containing electrotransformation buffer (HEB; 272 mM sucrose, 1 mM $MgCl_2$, 7 mM HEPES, pH 7.4). A single pulse at 25 µFD and 200 ohms at 12.5 KV/cm was used for electroporation. Clones were screened on APT agar containing 8 µg of erythromycin or 10 µg of chloramphenicol per ml, depending on the vector used. Colonies on the screening media were checked for bacteriocin activity. Bac⁺ clones were also screened directly on APT agar plates containing antibiotic and bacteriocin. These plates were prepared by growing *C. piscicola* LV17.C99 on 20 ml of APT agar for 12 h and overlayering with 20 ml of APT agar containing double the concentration of the appropriate antibiotic. Confirmation of clones was done by restriction enzyme analysis of the cloned DNA and by checking the bacteriocin activity produced by clones against a variety of indicator strains (Table 6.3, page 110) and immunity to other bacteriocin producing strains.

Religation study of pCP49

The purified *Bgl*II digest of pCP49 was ligated with T4 DNA ligase. The ligated DNA was electrotransformed into the chloramphenicol resistant strain of *C. piscicola* LV17.C2NZ by the procedure described above. Bac⁺ Cm^r transformants were screened on APT agar plates containing 10 µg chloramphenicol per ml and the bacteriocin produced by *C. piscicola* LV17.C99. Transformants were checked for their plasmid profiles and those showing reduced plasmid size were cured of pNZ12 by subculturing in the absence of chloramphenicol. The plasmid with reduced size was isolated and mapped for restriction sites.

6.3. Results

The results of the protoplast-induced curing of *C. piscicola* to obtain a mutant of *C. piscicola* LV17 containing only pCP49 is shown in Fig. 6.1. A strain designated *C. piscicola* LV17.C99, which had lost pCP9 and contained only pCP49 (Fig. 6.1, lane 3) was screened from the regeneration medium. Restriction analysis of pCP49 from *C. piscicola* LV17.C99 showed that no molecular change had occurred in pCP49 during the curing experiment (Fig. 6.1, lane 5). Purified pCP49 was mapped for its *Bgl*II sites with a partial digestion method (12; Fig. 6.2A). The physiological properties of the mutant strain LV17.C99, including carbohydrate fermentation patterns, sensitivity or resistance to 37 antibiotics, were the same as the parent strain, LV17.C33. The cross-activity and immunity between strain LV17.C99 containing pCP49 and strain LV17.TC3008M5 containing pCP40 are summarized in Table 6.2. Strain LV17.C99 produced the same amount of bacteriocin activity against the indicator strain *C. divergens* LV13 as the wild type strain LV17 containing both of the bacteriocin plasmids. The bacteriocin encoded by pCP49 is also active against TC3008M5 containing pCP40, but sensitive to the bacteriocin encoded by pCP40 and to the bacteriocins produced by wild type strain LV17. Therefore, it seems that bacteriocin encoded by pCP49 is different from the bacteriocin encoded by pCP40. The mutant strain *C. piscicola* LV17.C99 was used as the source of pCP49 DNA for further cloning experiments of the bacteriocin gene.

The 8.5 Kb chloramphenicol resistance-encoding plasmid pCaT from *L. plantarum* caTC2R, was used as vector DNA to clone the *Bgl*II fragments of pCP49. There are two *Bgl*II sites on pCaT, one at the end of a region encoding chloramphenicol resistance, the other outside of the chloramphenicol region (Fig. 6.2C, α and ß *Bgl*II sites, respectively). Ligation of the *Bgl*II digests of pCP49 and pCaT, and electrotransformation into *C. piscicola* LV17.C2 and UAL 26, resulted in the appearance of Bac$^+$, Cm^r colonies on the

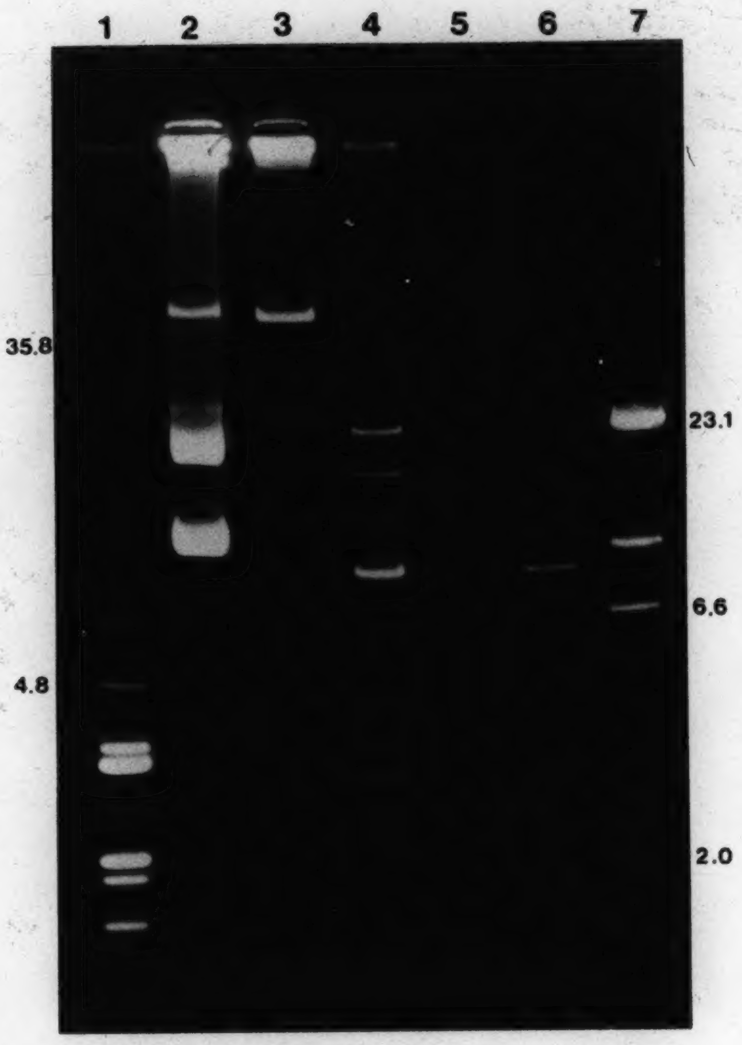

Fig. 6.1. Protoplast-induced curing of pCP9 from *C. piscicola* LV17.C33 containing pCP9 and pCP49.

Lane 1, mobility standard plasmids from *E. coli* V517; lanes 2 and 3, plasmid profile of *C. pisciola* LV17.C33 and LV17.C99, respectively; lanes 4 to 6 - *Bgl*II digestion pattern of plasmid(s) extracted from strain LV17.C33, LV17.C99, and LV17.C1, respectively.; lane 7 - *Hin*dIII, digests of bacteriophage λ DNA. Strain LV17.C1 contains pCP9 (2). Sizes of plasmids are expressed in megadalton, and sizes of fragment DNA is in kilobase.

Table 6.2. Interactions between *C. piscicola* strains producing bacteriocin(s) encoded by pCP40 and(or) pCP49. Producer strains were inoculated onto 20 ml M17 glucose agar plates, incubated 36 h, and overlayered with 10 ml of soft APT agar containing 10^7 CFU of indicator strain per ml. Diameters of the resulting zones of inhibition were measured in mm.

	Producer Strain of *C. piscicola*		
Indicator Strain	LV17	LV17.C99	LV17.TC3008M5
C. divergens LV13	29[a]	29	19
C. piscicola LV17	0	0	0
C. piscicola LV17.C99	10	0	8
C. piscicola LV17.TC3008M5	24	23	0

[a] Size of inhibitory zone, expressed in mm.

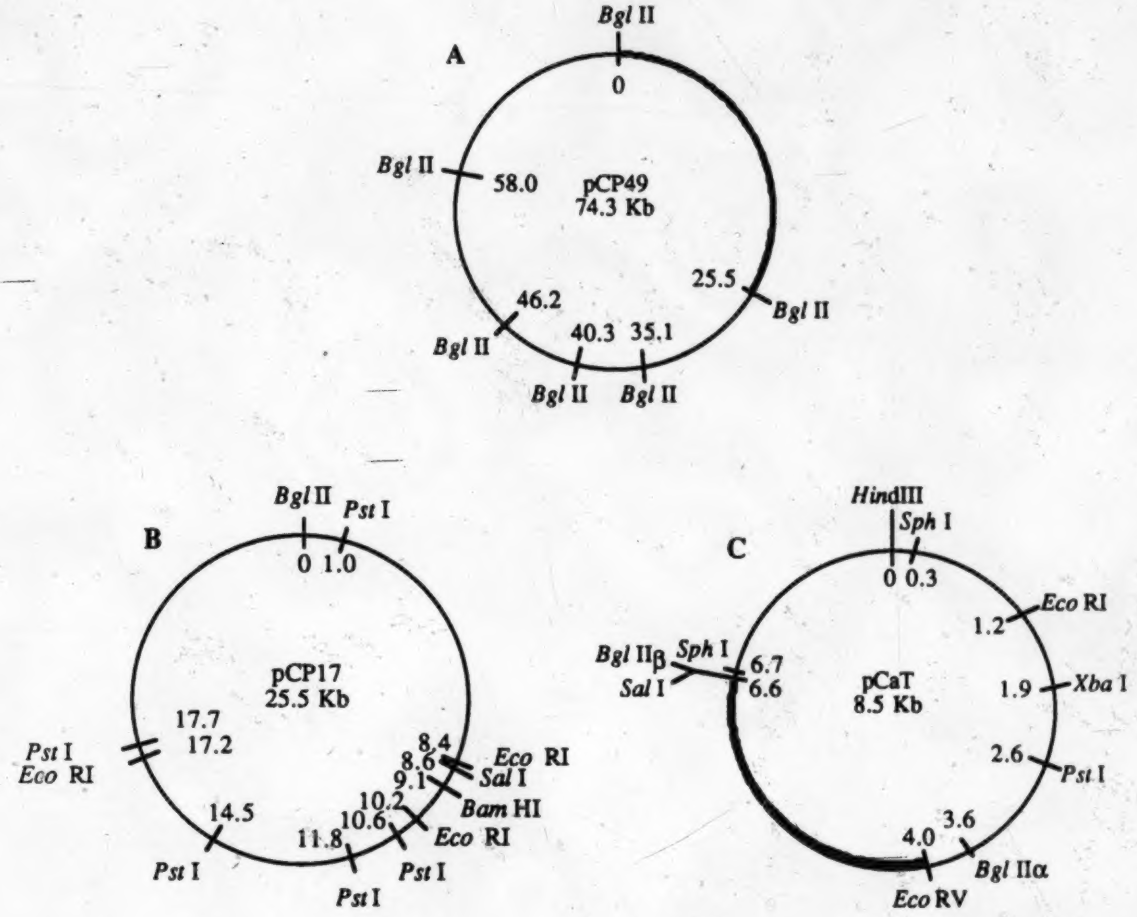
A
Bgl II
0
Bgl II
58.0
pCP49
74.3 Kb
25.5
Bgl II
46.2
Bgl II
40.3
35.1
Bgl II
Bgl II
B
Bgl II
Pst I
0 1.0
pCP17
25.5 Kb
17.7
17.2
Pst I
Eco RI
8.4
8.6
9.1
Eco RI
Sal I
Bam HI
10.2
10.6
Eco RI
14.5
11.8
Pst I
Pst I
Pst I
C
HindIII
Sph I
0 0.3
1.2
Eco RI
Bgl IIβ
Sph I
Sal I
6.7
6.6
pCaT
8.5 Kb
1.9
Xba I
2.6
Pst I
3.6
4.0
Bgl IIα
Eco RV

Fig. 6.2. Restriction maps of a bacteriocin plasmid pCP49 from *C. piscicola* LV17 (A), a religated plasmid pCP17 from a 25.5 Kb *Bgl*II fragment of pCP49 (B), and a chloramphenicol resistance plasmid pCaT from *L. plantarum* caTC2R (C).

A 25.5 Kb *Bgl*II fragment of pCP49 and a 2.6 Kb *Eco*RV - *Sal*I region containing chloramphenicol resistance gene of pCaT are marked as thick lines.

screening media containing 10 µg of chloramphenicol and bacteriocin produced by strain LV17.C99. Restriction analysis of the plasmid isolated from this colony showed that the 25.5 Kb fragment of pCP49 was inserted into one of the *Bgl*II sites of pCaT (Fig. 6.3, lanes 2 to 6). Further restriction analysis of the plasmid by *Pst*I and (or) *Bgl*II showed that the 25.5 Kb fragment was inserted into the ß site. The activity and specificity of bacteriocin produced by colonies containing this plasmid were the same as the parent strain, *C. piscicola* LV17.C99. Furthermore, when *C. piscicola* UAL26 was used as the recipient for cloned DNA, it showed retention of bacteriocin activity and specificity of the two bacteriocins, one encoded by the 25.5 Kb *Bgl*II fragment of pCP49 and the other encoded by the chromosome of UAL26, as shown in Table 6.3, producing the former bacteriocin in the early logarithmic phase of growth.

To determine the self-replicability of the 25.5 Kb *Bgl*II fragment of pCP49, the *Bgl*II digest of pCP49 was religated and transformed into *C. piscicola* LV17.C2NZ, which contains pNZ12d as a chloramphenicol resistance marker. Restriction analysis of DNA extracted from Bac$^+$ Cm^r transformants showed various kinds of smaller plasmids representing different combinations between the restriction fragments with 25.5 Kb fragment occurring in common or alone (Fig. 6.4). This confirms that the 25.5 Kb *Bgl*II fragment includes an origin of replication. Transformants containing the 25.5 Kb plasmid (pCP17; Fig. 6.4, lanes 3 and 5) were subcultured without chloramphenicol to destabilize the marker plasmid pNZ12d. The resulting Cm^s strain, *C. piscicola* LV17.C100 was used as a source of plasmid DNA for further subcloning studies of the 25.5 Kb fragment encoding bacteriocin activity and immunity in *C. piscicola* LV17. The restriction map of pCP17 is illustrated in Fig. 6.2.

Subcloning of a 16.7 Kb *Eco*RI fragment from 25.5 Kb Bac$^+$ Bac^r region of pCP49 using pCP17 as a plasmid source and pCaT as a vector resulted in subclones (strain F21, Fig. 6.5A) showing reduced bacteriocin activity against *C. divergens* LV13 (Table 6.4). The strain showed immunity to bacteriocin produced by itself, but it had almost the

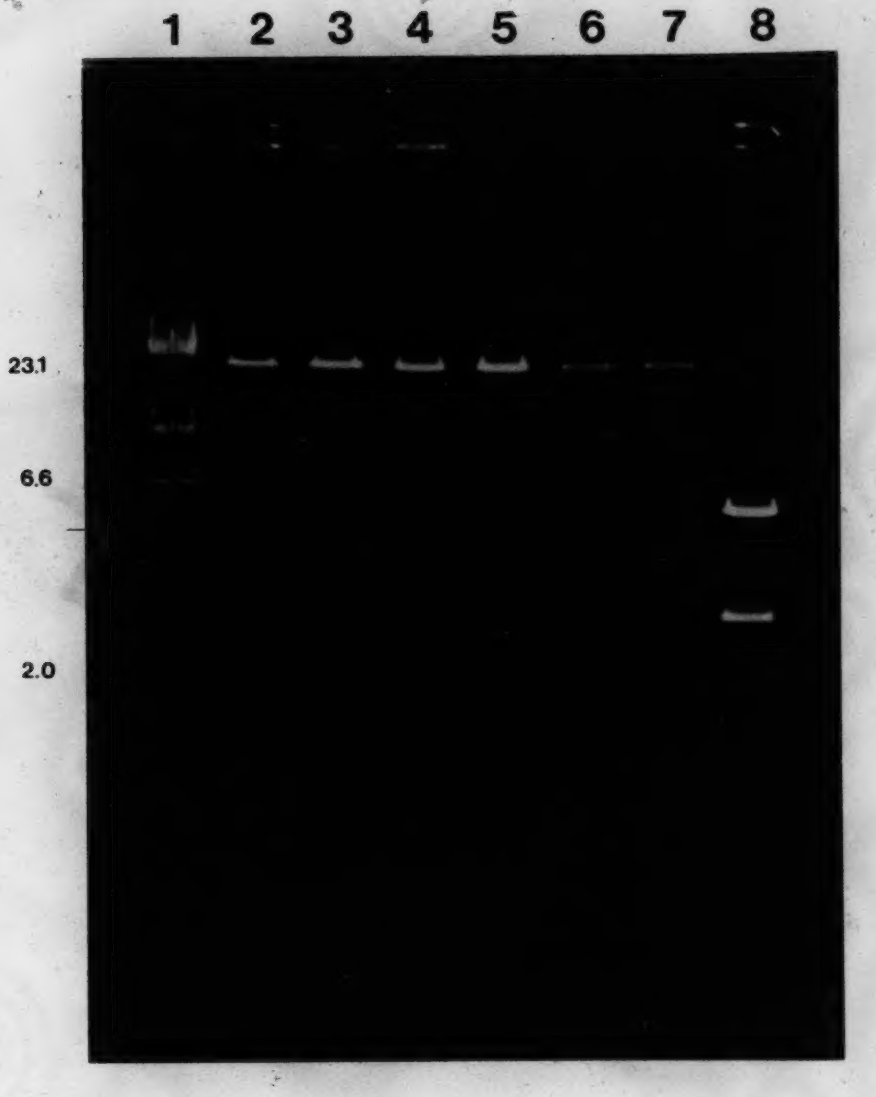

Fig. 6.3. Cloning of a 25.5 Kb *Bgl*II fragment of pCP49 into pCaT.

Lane 1 - *Hin*dIII digest of bacteriophage λ DNA; lanes 2 to 6 - *Bgl*II digests of cloned DNA; lanes 7 and 8 - *Bgl*II digests of pCP49 and pCaT, respectively. Size of fragment DNA is expressed in kilobases.

Table 6.3. Activity spectrum of cloned strain *C. piscicola* UAL26.CL97 containing a 25.5 Kb *Bgl*II fragment from a bacteriocin plasmid of *C. piscicola LV17*.

	Producer strain of *C. piscicola*		
Indicator strain	UAL26.CL97	UAL26	LV17.C99
Carnobacterium spp.			
C. piscicola LV17	-	-	-
C. piscicola UAL26	+[a]	-	+
C. divergens LV13	+	+	+
Lactobacillus spp.			
L. plantarum ATCC 4008	+	+	-
L. sake ATCC 15521	+	-	+
L. viridens ATCC 12706	+	+	+
Aciduric *Lactobacillus* strain UAL3	+	+	+
Aciduric *Lactobacillus* strain UAL12	+	+	+
Leuconostoc spp.			
Strain SML9	+	+	-
L. mesenteroides ATCC 23368	+	+	-
Pediococcus spp.			
P. acidilactici ATCC 8042	+	+	+
P. parvulus ATCC 19434	+	+	+
Enterococcus spp.			
E. faecalis ATCC 19433	+	-	+
E. faecium ATCC 19434	+	+	-
E. faecium (durans) ATCC 11576	+	+	+
Listeria spp.			
L. monocytogenes ATCC 15313	+	-	+
L. innocua ATCC 33090	-	-	-

[a] +, a diameter of clear zone larger than 5 mm; -, smaller than 5 mm.

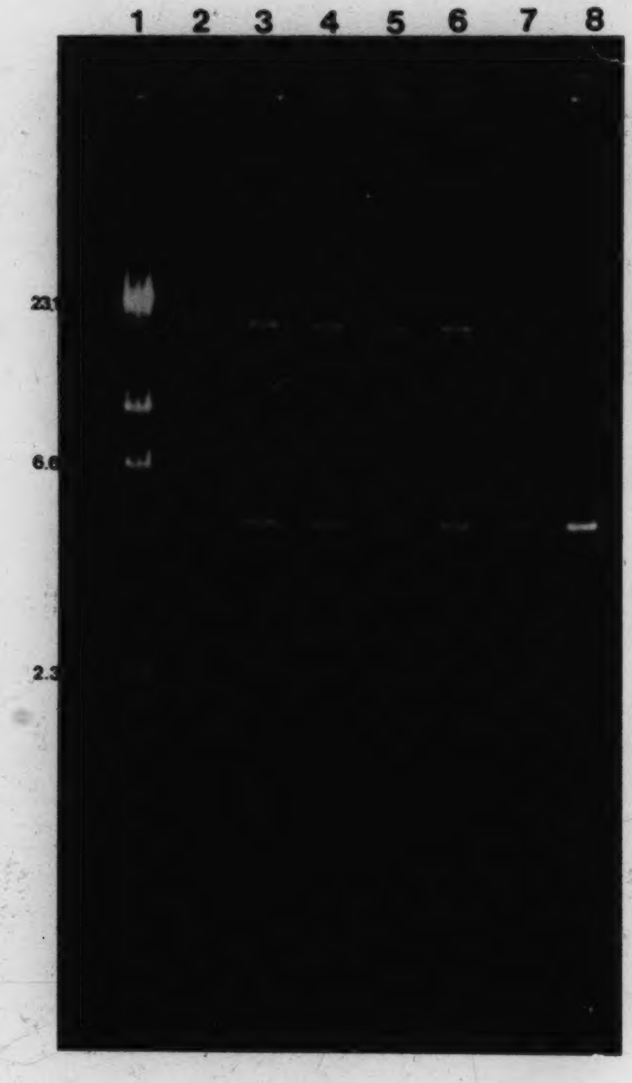

Fig. 6.4. *Bgl*II digestion pattern of plasmids originated from religated *Bgl*II fragments from pCP49.

Lane 1 - *Hin*dIII digest of bacteriophage λ DNA; lane 2 - *Bgl*II digest of pCP49; lanes 3 to 7 - *Bgl*II digests of religated plasmids; lane 8 - pNZ12d.

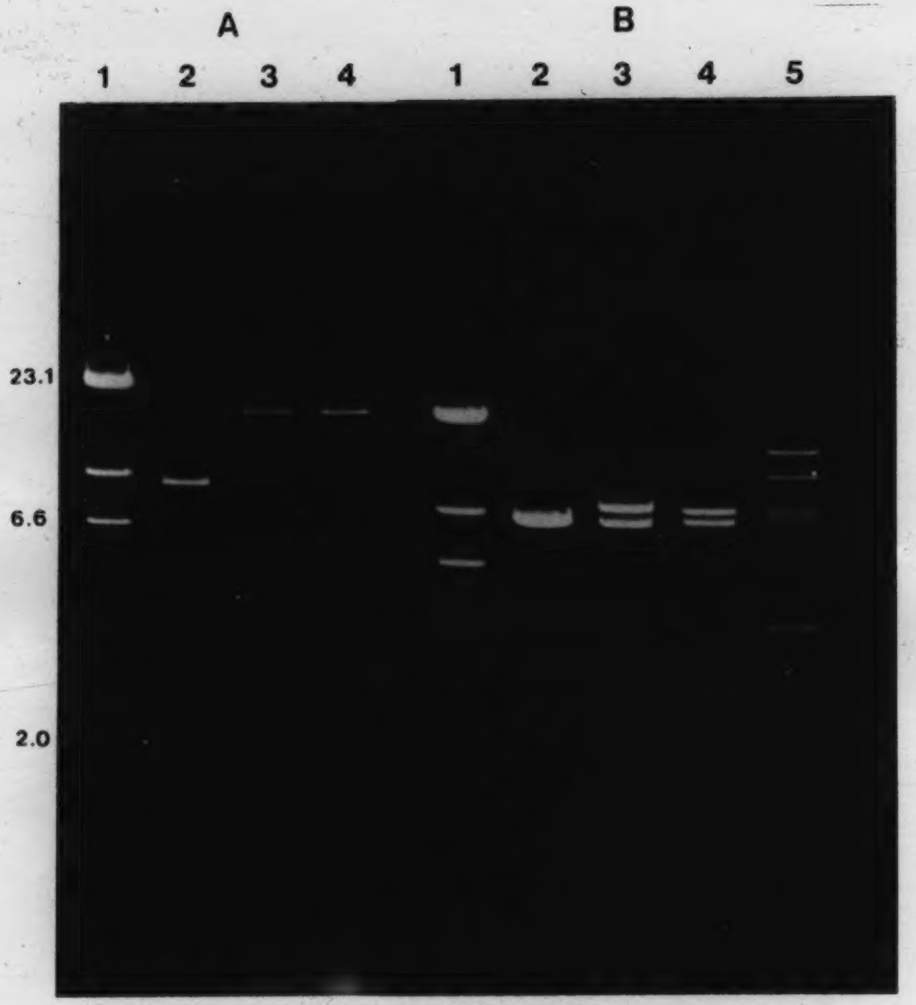

Fig. 6.5. Cloning of a 16.7 Kb *Eco*RI fragment (panel A) and a 9.6 Kb *Pst*I fragment of pCP17 (panel B) using pCaT as vector DNA.

Panel A: Lane 1 - *Hin*dIII digest of bacteriophage λDNA; lane 2 - pCaT digested by *Eco*RI; lane 3 - cloned DNA pCaT200 digested by *Eco*RI; lane 4 - pCP17 digested by *Eco*RI.

Panel B: Lane 1, *Hin*dIII digest of bacteriophage λ DNA; lane 2, pCaT digested by *Pst*I; lane 3, cloned DNA pCaT300 digested by *Pst*I; lane 4, pCP17 digested by *Pst*I; lane 5, pCP49 digested by *Pst*I.

Table 6.4. Bacteriocin activity and immunity of subclones of a 25.5 Kb *Bgl*II fragment from pCP49.

Indicator strain	LV17.C99[a] pCP49	LV17.F21 16.7 Kb *Eco*RI	LV17.G21 9.6 Kb *Pst*I	UAL26P263 5.4 Kb *Xba*I-*Pst*I	UAL26P264 pMG36e-CaT	UAL26R261 3.0 kb *Sph*I-*Pst*I
C. divergens LV13	+++[b]	++	+++	+	+	+
C. piscicola						
LV17.C99	-	-	-	-	-	-
UAL26	+++	+	+++	+	-	-
LV17.F21	++	-	+++	+	-	-
LV17.G21	-	-	-	-	-	-
UAL26 P263	-	-	-	-	-	-
UAL26 P264	+++	+	++	+	-	-
UAL26 R261	+	±	±	-	-	-

[a] Producer strains were inoculated onto 20 ml APT agar plates, incubated for 36 h, and overlayered with 10 ml of soft APT agar containing 10^7 CFU of indicator strain per ml.

[b] Diameter of clear zone was measured and expressed in mm; ±, 3-5 mm; +, 6-15 mm; ++, 16-25 mm; +++, 26-35 mm.

same sensitivity as *C. divergens* LV13 to bacteriocin produced by *C. piscicola* LV17.C99 which contains the Bac$^+$ plasmid pCP49 (Table 6.4). Therefore, it seems that the LV17 subclone F21 retains a limited capability to produce bacteriocin with loss of immunity.

However, subcloning of a 9.6 Kb *Pst*I fragment resulted in subclones (strain G21, Fig. 6.5B), which showed the same bacteriocin activity and immunity as strain LV17.C99, as shown in Table 6.4. The strain G21 was also active against subclone F21 containing the 16.7 Kb *Eco*RI fragment, and the specificity against various indicator strains was the same as strain LV17.C99 (data not shown).

The 9.6 Kb *Pst*I region of pCaT300 from subclone G21 (Fig. 6.6) was further studied by subcloning *Xba*I or *Sph*I fragments of pCaT300 into the corresponding site of pMG36e. When the 13.2 Kb *Xba*I fragment (*Xba*I$_A$-*Xba*I$_B$ of pCaT300, Fig. 6.6) which contained 5.4 Kb *Xba*I$_A$-*Pst*I$_B$ fragment of 9.6 Kb Bac$^+$ Bac^r region was cloned into pMG36e (Fig. 6.7A, lane 3), the resultant subclone (UAL26 P263) showed reduced bacteriocin activity, but complete immunity to bacteriocin produced by strain LV17.C99 and subclone G21 (Table 6.4). Another subclone (UAL26 P264) containing the 7.8 Kb *Pst*I-*Xba*I$_B$ fragment of pCaT in pCaT300 (Fig. 6.7, lane 2 and 4) showed no bacteriocin activity and no immunity to bacteriocin produced by strain LV17.C99. Clones containing the 4.2 Kb *Pst*I$_A$-*Xba*I$_A$ fragment of the 9.6 Kb Bac$^+$ Bac^r were not found.

Subcloning of *Sph*I fragment of pCaT300 into pMG36e resulted in a clone (UAL26 R261) containing a 7.1 Kb fragment (Fig. 6.7B) which has 3.0 Kb *Sph*I$_B$-*Pst*I$_B$ fragment of the 9.6 Kb Bac$^+$ Bac^r region. This subclone R261 did not exhibit any bacteriocin activity but showed almost complete immunity to bacteriocin produced by strain LV17.C99 or subclone G21 (Table 6.4). The results of the cloning experiments are summarized in Fig. 6.8.

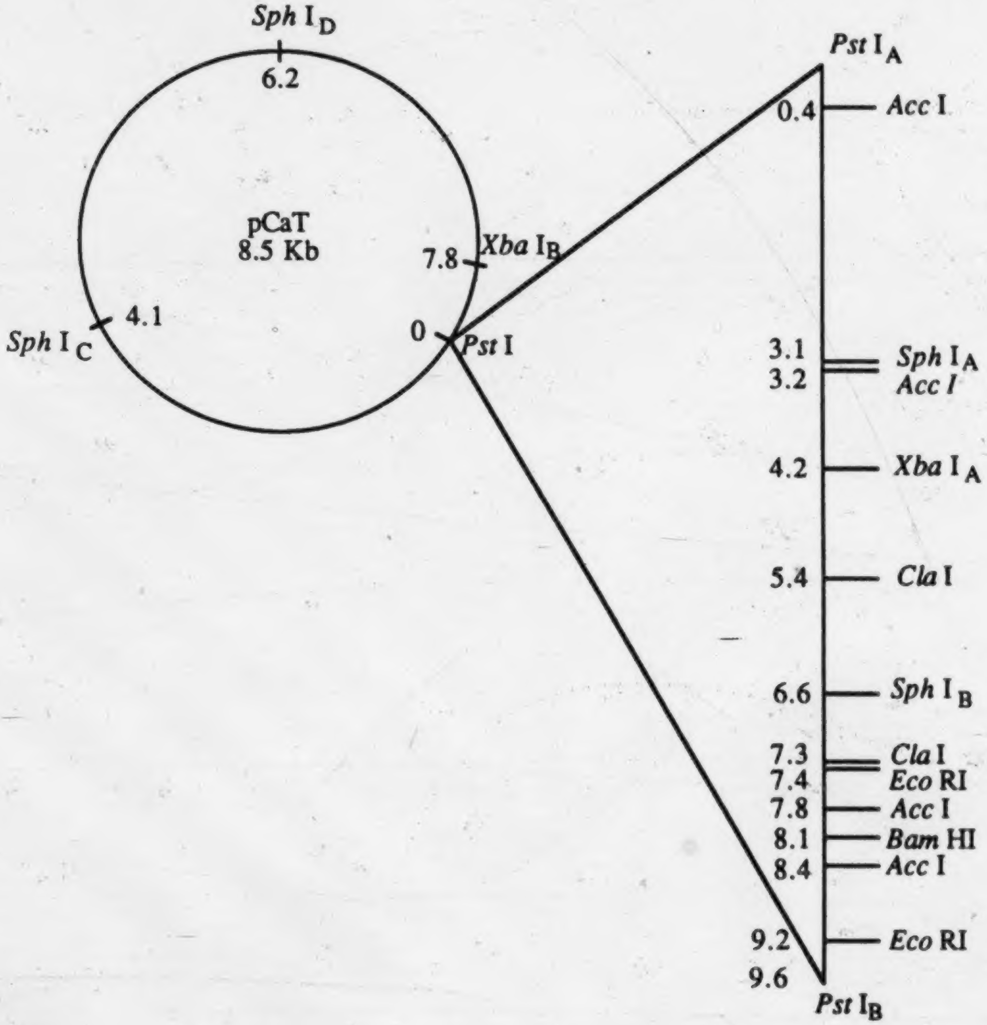
Sph I D
6.2
pCaT
8.5 Kb
7.8
Xba I B
4.1
Sph I C
0
Pst I
Pst I A
0.4
Acc I
3.1
Sph I A
3.2
Acc I
4.2
Xba I A
5.4
Cla I
6.6
Sph I B
7.3
Cla I
7.4
Eco RI
7.8
Acc I
8.1
Bam HI
8.4
Acc I
9.2
Eco RI
9.6
Pst I B

Fig. 6.6. Restriction map of pCaT300 containing a 9.6 Kb fragment from pCP17.

Subscripts A, B, C, or D are used to differentiate the restriction sites of the same restriction enzymes.

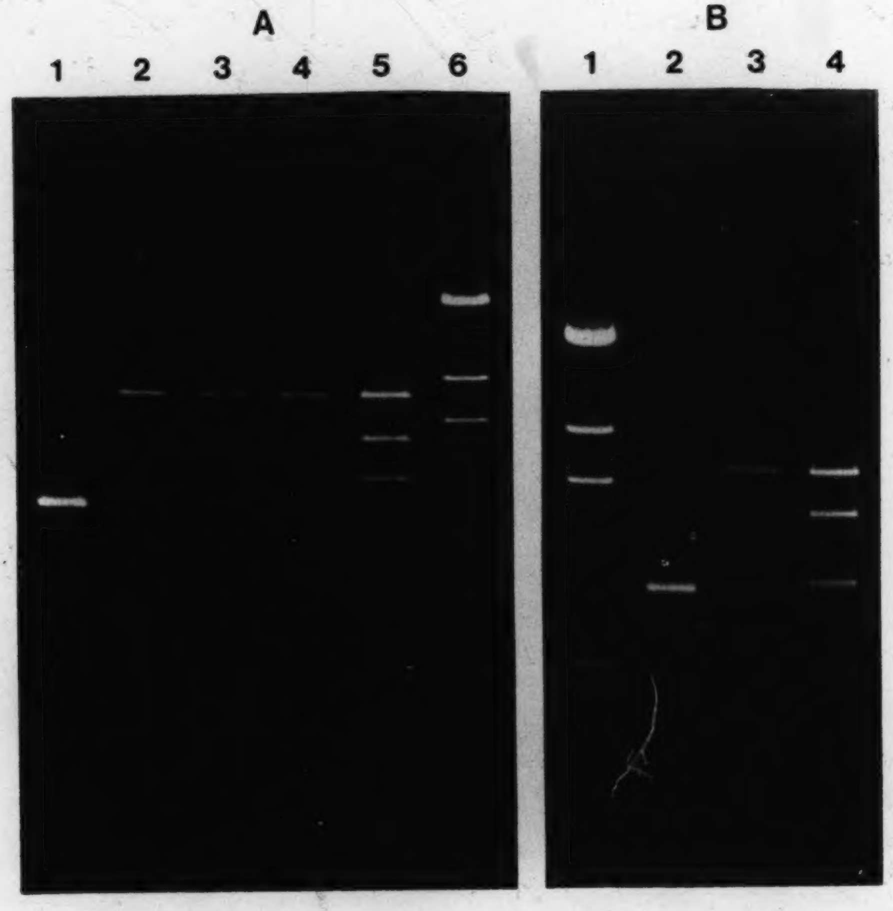

Fig. 6.7. Subcloning of a 9.6 Kb *Pst*I region of pCaT300 using pMG36e as vector DNA.

A. *Pst*I-*Xba*I double digestion of pMG36e (lane 1), cloned DNA from subclone P261 (lane 2), P263 (lane 3), P264 (lane 4), pCaT 300 (lane 5), *Hin*dIII digest of bacteriophage λ DNA (lane 6).

B. *Sph*I - digestion of pMG36e (lane 2), cloned DNA from subclone R261 (lane 3), pCaT 300 (lane 4); *Hin*dIII digest of bacteriophage λ DNA (lane 1).

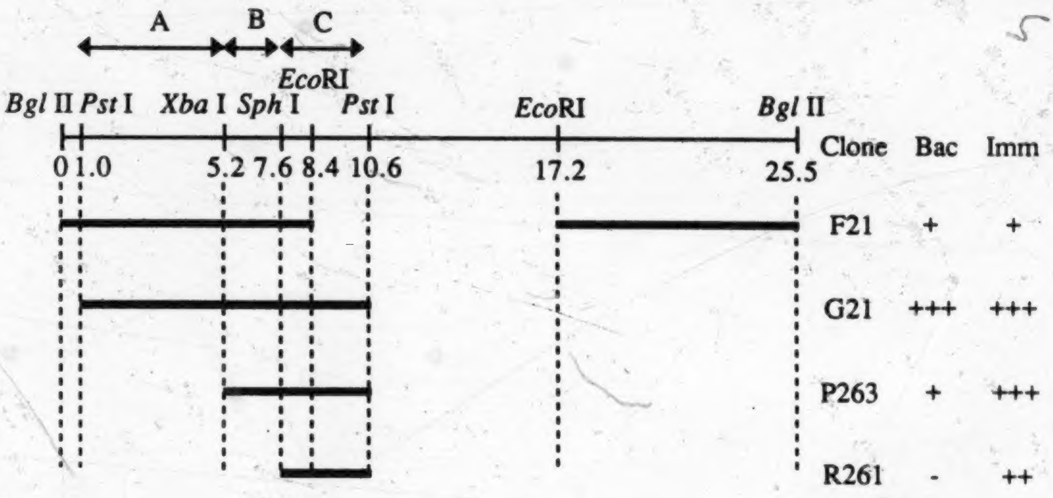
A
B
C
*Eco*RI
Bgl II
Pst I
Xba I
Sph I
Pst I
*Eco*RI
Bgl II
0
1.0
5.2
7.6
8.4
10.6
17.2
25.5
Clone
Bac
Imm
F21
+
+
G21
+++
+++
P263
+
+++
R261
-
++

Fig. 6.8. Summary of cloning experiments.

Cloned regions are shown as thick bars. Bacteriocin activity (Bac) and immunity (Imm) to the bacteriocin are shown at the right of the diagram. Bac or Imm comparable to that of *C. piscicola* LV17.C99 are expressed as +++. Parts A, B, and C are marked for discussion.

6.4. Discussion

Results of cloning experiments for genes encoding bacteriocin production and immunity to the bacteriocin in a plasmid pCP49 from *C. piscicola* LV17 showed that a 9.8 Kb *Pst*I fragment from the plasmid contains the Bac and Imm genes. By further cloning experiments with the *Eco*RI fragment from pCP17 and subcloning experiments of the *Xba*I-*Sph*I fragment from the 9.8 Kb *Pst*I fragment-containing clone, the 9.8 Kb Bac^+ Bac^r region could be divided into three parts which are responsible for bacteriocin production or immunity to the bacteriocin (Fig. 6.8, parts A,B, and C).

The gene(s) encoding bacteriocin activity of pCP49 seems to reside on parts A and B, because deletion of part A reduced bacteriocin activity significantly in subclone P263, and deletion of both parts A and B in subclone R261 rendered the strain nonbacteriocinogenic. However, considering the fact that even the largest bacteriocin molecule which has been reported so far has 37,500 dalton (8) and it requires only a 0.9 Kb length of DNA for its structural gene, part A (4.2 Kb) and part B (2.4 Kb) are too large to contain only the gene(s) encoding bacteriocin production. Therefore it is probable that (i) part A contains regulatory elements, including the promoter for bacteriocin production and part B encodes structural genes for the bacteriocin; or (ii) the regulatory elements reside on part A which is followed by multiple copies of the bacteriocin gene encompassing parts A and B; or (iii) multicopies of the bacteriocin gene each with its own promoter are present throughout parts A and B of the Bac^+ region. In hypotheses (i) and (ii), the reduction of bacteriocin activity in subclone P263 can be explained by the lack of a promoter which is more effectively recognized by *Carnobacterium* than the P32 promoter of pMG36e. In hypothesis (iii), reduced bacteriocin activity may be explained by the reduced gene dosage effect.

Van Belkum *et al.* (16) reported that the bacteriocin and resistance genes of *Lactococcus lactis* subsp. *cremoris* have an operon-like structure, with two or three copies of bacteriocin and resistance genes following the common promoter. This supports the first and second possibilities described above. However, to confirm these assumptions, it would be necessary to clone part A or B separately into other host strains which are resistant to the bacteriocin of *C. piscicola* LV17.C99 and capable of processing the bacteriocin gene product.

Part C seems to be responsible for immunity to bacteriocin encoded by pCP49, because subclone R261 containing only part C (3.0 Kb *Sph*I-*Pst*I region) showed almost complete immunity to bacteriocin produced by strain LV17.C99 containing pCP49 and subclones containing parts A and B, but did not show any bacteriocin activity. This conclusion is further supported by the sensitivity of subclone F21 to the bacteriocin. This clone lacks the 2.1 Kb *Eco*RI-*Pst*I fragment of part C. However, considering that subclone P263 shows complete immunity to the bacteriocin produced by strain LV17.C99, the Imm gene(s) starts a little upstream of part C. The presence of vector DNA, pCaT or pMG36e, did not have any relationship with immunity to the bacteriocin as evidenced by subclone P264 (Table 6.4) which contains the recombinant vector comprised of pCaT and pMG36e.

The reason why subclone F21 showed reduced bacteriocin activity is not clear, because it seems to contain all of parts A and B from the Bac$^+$ gene region. However, it is probably that the lack of complete immunity resulting from partial absence of part C disrupts the normal intracellular processing of the bacteriocin gene product. Alternatively, the amount of bacteriocin accumulated in the medium is reduced due to the reduction of the number of bacteriocin-producing cells, because of a suicidal effect of bacteriocin on the producer cells. In *L. lactis* subsp. *cremoris* it was observed that bacteriocin producing strains which lack immunity genes gain physiological resistance to the bacteriocin produced by themselves, but show very poor growth (Van Belkum, personal communication).

The chloramphenicol resistance plasmid pCaT was useful as an intermediate vector in *Carnobacterium*, with a high transformation efficiency and intactness of the molecules during electroporation. Although it lacks unique restriction sites in the chloramphenicol resistance gene or an alternate marker, it is useful for cloning genes that encode a selectable marker which can be screened for directly on the selective agar plates, as in the case of bacteriocin (10). The pCaT plasmid containing the Bac region from pCP49 was also transformed into another strain of *C. piscicola*, UAL26, which produces a chromosomally determined bacteriocin. Both of the bacteriocins were expressed in *C. piscicola* UAL26.CL97, giving the recipient cell a broader spectrum of antibacterial activity. The combined effects of two bacteriocins derived from two different species would be one example of improving antibiosis of lactic starter cultures against spoilage microorganisms, while contributing to the desirable quality of fermentation and prolonged shelf-life of the fermented foods. Another genetic approach to this goal would be to engineer the bacteriocin gene to produce more active bacteriocin by changing its molecular structure, or to combine several genes into one plasmid or chromosomal region to produce "super bacteriocins" as proposed by Klaenhammer (10). However, with regard to the bacteriocin produced by *C. piscicola* LV17, more information is required on (i) genetic structure of structural gene and regulatory elements, including the analysis of the nucleotide sequence, and (ii) expression and post-translational events of the gene product.

6.5. References

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7. General Conclusions of the Study

The objectives of this research were to characterize the antagonistic substance produced by *C. piscicola* LV17 with respect to its physiological, biochemical, and genetic characteristics, in order to establish a scientific basis for the possible future application of the bacterium or its antagonistic substance to foods. The antagonistic substance was shown to be a bacteriocin. This is the first bacteriocin characterized in *Carnobacterium*, and it extends our current understanding of bacteriocins produced by lactic acid bacteria. However, not all of the initial questions were answered in this study, leaving considerable scope for further research.

7.1. Physiological aspects of bacteriocin production by *C. piscicola* LV17

C. piscicola LV17 produces two bacteriocins mediated by two different plasmids, pCP40 and pCP49. The characteristic early detection of bacteriocin activity observed in the early logarithmic growth phase of wild type strain was attributed to the bacteriocin encoded by pCP49. The bacteriocin encoded by pCP40 was only detected in the stationary phase. Early production of bacteriocin could be a physiological advantage to the producer strain in competition with other microorganisms in the meat microflora. The early production of bacteriocin may compensate for the handicaps of a nonaciduric lactic acid bacterium, such as *C. piscicola* LV17, enabling it to become a significant part of the meat microflora despite its pH dependence. The biological reason for, or mechanism of, early production of bacteriocin is unknown. However, the determinant that links production of bacteriocin to the early growth cycle of cells seems to reside in the Bac locus of pCP49, because another *C. piscicola* strain UAL26, which produces chromosomally determined bacteriocin in the late logarithmic to early stationary phase, produces the bacteriocin of *C. piscicola* LV17 early in the growth cycle when it receives the Bac locus. However, it is possible that early production is an intrinsic property of the bacteriocin molecule itself.

The physiological meaning of producing two bacteriocins encoded by two different plasmids in one strain is not clear, because maintaining two large plasmids with one of them encoding a bacteriocin whose activity spectrum is just a part of the other's activity spectrum would be energetically and teleologically unfavorable. Further studies on the origin of two bacteriocin genes with respect to genetic homology would be interesting.

7.2. Biochemical aspects of bacteriocins of *C. piscicola* LV17

The molecular weights of both bacteriocins produced by *C. piscicola* LV17 seem to be greater than 10,000 daltons, because both of them are retained by dialysis tubing with a molecular weight cut-off of 6,000 to 8,000 daltons and on a filter membrane with molecular weight cut-off of 10,000 daltons. Additional data on the bacteriocins obtained during purification trials (not presented in the thesis because of the loss of activity during the final steps of purification and because of streaking on the gel electrophoretograms) indicates that they are present as aggregates or conjugates with other stabilizing molecules. The bacteriocins are considered to be hydrophobic, because both bind to phenyl sepharose gel when eluted with ammonium sulfate or organic solvents. Stability of the bacteriocins during heating, and rapid renaturing ability of bacteriocins after exposure to extremes of pH support the probability that they require stabilizing conjugates, or the presence of intramolecular stabilizing reagents such as disulfide bridges. However, further purification is required to assess the exact molecular nature of these bacteriocins and to differentiate between the two bacteriocins.

The bactericidal mode of inhibition was not evaluated for each of the bacteriocins from *C. piscicola* LV17. However, the bactericidal action exerted by two bacteriocins requires intact structural contact between the cell wall and cytoplasmic membrane. The bacteriocins act on the cytoplasmic membrane. The exact mechanism of action requires more research to determine whether the lethal effect of the bacteriocin molecules results from penetration of the cytoplasmic membrane or location of the bacteriocins in the transmembrane area. Because of their bactericidal effect, it is concluded that the target site is one of essential biological function of target cells, including macromolecular synthesis, energy metabolism, or depolarization of cell membrane. This also requires further study to evaluate each possibility.

7.3. Genetic aspects of bacteriocin production in *C. piscicola* LV17

Genes encoding bacteriocin production mediated by pCP49 were extensively studied. The 9.6 Kb region of pCP49 has a locus responsible for bacteriocin production, immunity to the bacteriocin, and possible linkage to the early logarithmic phase in the growth cycle of the cell. Results of the subcloning experiments indicate that the locus might have an operon-like structure with regulatory elements for transcription and translation in a 4.2 Kb region, multiple copies of the structural gene in a 2.4 Kb region, and immunity gene in a 3.0 Kb region. However, further cloning and sequencing experiments should be done to determine the entire genetic structure of the bacteriocin locus, and to understand the genetic mechanism of expression and regulation of these genes. Considering that the 25.5 Kb *Bgl*II fragment of pCP49 also contains a junction fragment, which is generated during co-integrate formation, it is possible that the bacteriocin locus contains an insertional sequence in upstream or downstream regions of the probable bacteriocin operon.

The genetic basis for immunity to bacteriocin is not known for gram-positive bacteriocins, except for the nisin resistance gene (2, 6). In this context, it would be valuable to perform *in vivo* complementation tests between a subclone which does not contain the immunity region and a subclone which contains the immunity gene, in order to assess whether there is a *cis*- or *trans*-acting effect between those two cloned DNA's.

7.4. Applicability of methods and products

Several methods were applied in this study which may be of value for use in related studies. The plasmid extraction procedure, which was derived from Klaenhammer (7) and Birnboim (1), proved to be valuable in extracting plasmids larger than 40 MDa. The protoplast-induced curing method, modified from Gasson (4), also proved useful in separating residential plasmids from each other, especially where chemical methods with mutagenic compounds or antibiotics failed to cure or separate the plasmids.

The pAMβ1-associated mobilization technique has been recommended to transfer nonconjugative plasmids (3). It proved useful for tranferring nonconjugative bacteriocin plasmids in *C. piscicola* and separating pCaT in *L. plantarum* from other residential plasmids. Furthermore, the instability of the plasmid pAMβ1 in the absence of antibiotic pressure or resistance to electrotransformation could be exploited to remove the mobilizing plasmid from the genetically modified microorganisms. This provides a way in which strains can be improved by introducing intact plasmids encoding useful properties without *in vitro* treatment.

The possibility of using pCaT as an intermediate vector DNA, especially in *Carnobacterium* is promising because of its high electrotransformability and intact expression in both *C. piscicola* and *C. divergens*. Further development of pCaT as a shuttle vector between different Gram-positive genera or between Gram-positive and Gram-negative bacteria would be possible by introducing different origins of replication and selectable markers into the vector. In this context, pCP17, which has reduced size by religation of *Bgl* II fragments of pCP49, but has its own replication origin and the 3.0 Kb immunity gene region of the bacteriocin plasmid, would be a useful material for creating a food-grade vector (9) for strain improvement of lactic acid bacteria.

As shown with strain UAL26.CL97, combining two different bacteriocin-producing abilities in one host strain gave an accumulative activity spectrum against sensitive strains, proceeding one step towards a 'superbacteriocin' (5). Alternatively, a defined gene area of a bacteriocin plasmid of *C. piscicola* LV17 could serve as one of many bacteriocin gene cassettes which can be readily combined to give desirable specificity spectra.

7.5. Applicability of bacteriocins and bacteriocin-producing strains to the food industry

Prospects of using bacteriocins produced by *C. piscicola* LV17 as food preservatives or using a bacteriocinogenic strain as a meat starter culture with competitive advantage over other microorganisms in meat are hard to predict. *In vivo* experiments on the effect of genetically engineered strain UAL26.CL97 on the microflora of cooked meats are in progress in our laboratory. Considering the ambiguities that have occurred with many bacteriocins (8, 10) in translating *in vitro* experimental results into *in vivo* situations, careful assessment and gradual solution of the practical problems should be sought in parallel with *in vitro* basic research.

7.6. References

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